(51) **International Patent Classification:**
C12Q 1/68 (2006.01)   C12P 19/34 (2006.01)

(21) **International Application Number:**
PCT/US2005/013883

(22) **International Filing Date:** 22 April 2005 (22.04.2005)

(25) **Filing Language:** English

(26) **Publication Language:** English

(30) **Priority Data:**
60/566,967   29 April 2004 (29.04.2004)   US

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(81) **Designated States** *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SM, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:
— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) **Title: MASS TAG PCR FOR MULTIPLEX DIAGNOSTICS**

(57) **Abstract:** This invention provides a mass tag-based method for simultaneously detecting in a sample the presence of one or more of a plurality of different target nucleic acids. This invention also provides related kits.

MASS TAG PCR FOR MUTLIPLEX DIAGNOSTICS

5   This application claims priority of U.S. Provisional Application No. 60/566,967, filed April 29, 2004, the contents of which are hereby incorporated by reference.

Throughout this application, various publications are
15   referenced. Full citations for these references may be found at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application to more fully describe
20   the state of the art to which this invention pertains.

Background of the Invention

Establishing a causal relationship between infection with
25   a virus and a specific disease may be complex. In most acute viral diseases, the responsible agent is readily implicated because it replicates at high levels in the affected tissue at the time the disease is manifest, morphological changes consistent with infection are
30   evident, and the agent is readily cultured with standard microbiological techniques. In contrast, implication of viruses in chronic diseases may be confounded because persistence requires restricted gene expression, classical hallmarks of infection are absent, and/or
35

mechanisms of pathogenesis are indirect or subtle.

Methods for cloning nucleic acids of microbial pathogens
directly from clinical specimens offer new opportunities
to investigate microbial associations in chronic
diseases. The power of these methods is that they can
succeed where methods for pathogen identification through
serology or cultivation may fail due to absence of
specific reagents or fastidious requirements for agent
replication. Over the past decade, the application of
molecular pathogen discovery methods resulted in
identification of novel agents associated with both acute
and chronic diseases, including Borna disease virus,
Hepatitis C virus, Sin Nombre virus, HHV-6, HHV-8,
Bartonella henselae, and Tropherema whippeli.

Various methods are employed or proposed for cultivation-
independent characterization of infectious agents. These
can be broadly segregated into methods based on direct
analysis of microbial nucleic acid sequences (e.g., cDNA
microarrays, consensus PCR, representational difference
analysis, differential display), direct analysis of
microbial protein sequences (e.g., mass spectrometry),
immunological systems for microbe detection (e.g.,
expression libraries, phage display) and host response
profiling. A comprehensive program in pathogen discovery
would need to exploit most, if not all, of these
technologies.

The decision to employ a specific method is guided by the
clinical features, epidemiology, and spectrum of
potential pathogens to be implicated. Expression

libraries, comprised of cDNAs or synthetic peptides, may be useful tools in the event that large quantities of acute and convalescent sera or cerebrospinal fluid are available for screening purposes; however, the approach

5   is cumbersome, labor-intensive, and success is dependent on the presence of a specific, high affinity humoral immune response. The utility of host response mRNA profile analysis has been demonstrated in several *in vitro* paradigms and some inbred animal models;

10   nonetheless, it is important to formally consider the possibility that a variety of organisms may activate similar cascades of chemokines, cytokines, and other soluble factors that influence host gene expression to produce what are likely to be convergent gene expression

15   profiles. Thus, at least in virology, it is prudent to explore complementary methods for pathogen identification based on agent-encoded nucleic acid motifs. Given the potential for high density printing of microarrays, it is feasible to design slides or chips decorated with both

20   host and pathogen targets. This would provide an unprecedented opportunity to simultaneously survey host response mRNA profiles and viral flora, providing insights into microbial pathogenesis not apparent with either method of analysis alone.

25

Representational difference analysis (RDA) is an important tool for pathogen identification and discovery. However, RDA is a subtractive cloning method for binary comparisons of nucleic acid populations. Thus, although

30   ideal for analysis of cloned cells or tissue samples that differ only in a single variable of interest, RDA is less well suited to investigation of syndromes wherein

infection with any of several different pathogens results in similar clinical manifestations, or infection is not invariably associated with disease. An additional caveat is that because the method is dependent upon the presence

5   of a limited number of restriction sites, RDA is most likely to succeed for agents with large genomes. Indeed, in this context, it is noteworthy that the two viruses detected by RDA in the listing above were herpesviruses.

10   Consensus PCR (cPCR) has been a remarkably productive tool for biology. In addition to identifying pathogens, particularly genomes of prokaryotic pathogens, this method has facilitated identification of a wide variety of host molecules, including cytokines, ion channels, and

15   receptors. Nonetheless, until recently, a difficulty in applying cPCR to pathogen discovery in virology has been that it is difficult to identify conserved viral sequences of sufficient length to allow cross-hybridization, amplification, and discrimination using

20   traditional cPCR format. While this may not be problematic when one is targeting only a single virus family, the number of assays required becomes infeasible when preliminary data are insufficient to allow a directed, limited analysis.

25

Real-time PCR methods have significantly changed diagnostic molecular microbiology by providing rapid, sensitive, specific tools for detecting and quantitating genetic targets. Because closed systems are employed,

30   real-time PCR is less likely than nested PCR to be confounded by assay contamination due to inadvertent aerosol introduction of amplicon/positive control/cDNA

templates that can accumulate in diagnostic laboratories. The specificity of real time PCR is both a strength and a limitation. Although the potential for false positive signal is low so is the utility of the method for

5   screening to detect related but not identical genetic targets. Specificity in real-time PCR is provided by two primers (each approximately 20 matching nucleotides (nt) in length) combined with a specific reporter probe of about 27 nt. The constraints of achieving hybridization

10   at all three sites may confound detection of diverse, rapidly evolving microbial genomes such as those of single-stranded RNA viruses. These constraints can be compensated in part by increasing numbers of primer sets accommodating various templates. However, because real-

15   time PCR relies on fluorescent reporter dyes, the capacity for multiplexing is limited to the number of emission peaks that can be unequivocally separated. At present up to four dyes can be identified simultaneously. Although the repertoire may increase, it will not likely

20   change dramatically.

Summary of the Invention

This invention provides a method for simultaneously detecting in a sample the presence of one or more of a plurality of different target nucleic acids comprising the steps of:

(a)   contacting the sample with a plurality of nucleic acid primers simultaneously and under conditions permitting, and for a time sufficient for, primer extension to occur, wherein (i) for each target nucleic acid at least one predetermined primer is used which is specific for that target nucleic acid, (ii) each primer has a mass tag of predetermined size bound thereto via a labile bond, and (iii) the mass tag bound to any primer specific for one target nucleic acid has a different mass than the mass tag bound to any primer specific for any other target nucleic acid;

(b)   separating any unextended primers from any extended primers;

(c)   simultaneously cleaving the mass tags from any extended primers; and

(d)   simultaneously determining the presence and sizes of any mass tags so cleaved,

wherein the presence of a cleaved mass tag having the same size as a mass tag of predetermined size previously bound to a predetermined primer indicates the presence in the sample of the target nucleic acid specifically recognized by that predetermined primer.

This invention further provides the instant method, wherein the method detects the presence in the sample of

10 or more, 50 or more, 100 or more, or 200 or more different target nucleic acids. This invention further provides the instant method, wherein the sample is contacted with 4 or more, or 10 or more, or 50 or more, or 100 or more, or 200 or more different primers.

This invention further provides the instant method, wherein one or more primers comprises the sequence set forth in one of SEQ ID NOs:1-96, and 98-101. This invention further provides the instant method, wherein at least two different primers are specific for the same target nucleic acid. This invention further provides the instant method, wherein a first primer is a forward primer for the target nucleic acid and a second primer is a reverse primer for the same target nucleic acid.

This invention further provides the instant method, wherein the mass tags bound to the first and second primers are of the same size. This invention further provides the instant method, wherein the mass tags bound to the first and second primers are of a different size.

This invention further provides the instant method, wherein at least one target nucleic acid is from a pathogen.

This invention further provides the instant method, wherein the presence and size of any cleaved mass tag is determined by mass spectrometry. This invention further provides the instant method, wherein the mass spectrometry is selected from the group consisting of atmospheric pressure chemical ionization mass

spectrometry, electrospray ionization mass spectrometry, and matrix assisted laser desorption ionization mass spectrometry.

Brief Description of the Figures

Figure 1: This figure shows the structure of mass tag precursors and four photoactive mass tags.

5

Figure 2: This figure shows an ACPI mass spectrum of mass tag precursors for digital virus detection.

Figure 3: This figure shows DNA sequencing sample
10 preparation for MS analysis using biotinylated dideoxynucleotides and a streptavidin coated solid phase.

Figure 4: This figure shows a mass spectrum from Sanger sequencing reactions using dd(A, G, C)TP-11-biotin and
15 ddTTP-16-biotin.

Figure 5: This figure shows synthesis of NHS ester of one mass tag for tagging amino-primer (SEQ ID NO:97).

20 Figure 6: This figure shows the general structure of mass tags and photocleavage mechanism to release the mass tags from DNA for MS detection.

Figure 7: This figure shows four mass tagged biotinylated
25 ddNTPs.

Figure 8: This figure shows the structure of four mass tag precursors and the four photoactive mass tags.

30 Figure 9: This figure shows APCI mass spectra for four mass tags after cleavage from primers. 2-nitrosacetophenone, m/z 150; 4 fluoro-2-

nitrosacetophenone,   m/z   168;   5-methoxy-2-

nitrosacetophenone,   m/z   180;   and   4,5-dimethoxy-2-

nitrosacetophenone.

5   Figure 10: This figure shows four mass tag-labeled DNA

molecules.

Figure 11: This figure shows differential real-time PCR

for HCoV SARS, OC43, and 229E.

10

Figure 12: This figure shows 58 tags cleaved from

oligonucleotides and detected using ACPI-MS. Each peak

represents a different tag structure as a unique

signature of the oligonucleotide it was originally

15   attached to.

Figure 13: This figure shows singleplex mass tag PCR for

(1) influenza A virus matrix protein, (2) human

coronavirus SARS, (3) 229E, (4) OC43, and (5) the

20   bacterial agent M. pneumoniae. (6) shows a 100bp ladder.

Figure 14: This figure shows mass spectrum representative

of data collected using a miniaturized cylindrical ion

trap mass analyzer coupled with a corona discharge

25   ionization source.

Figure 15: This figure shows mass spectrum of perfluoro-

dimethylcyclohexane collected on a prototype atmospheric

sampling glow discharge ionization source.

30

Figure 16: This figure shows the sensitivity of a 21-plex

mass tag PCR. Dilutions of cloned gene target standards

(10 000, 1 000, 500, 100 molecules/assay) diluted in human placenta DNA were analyzed by mass tag PCR. Each reaction mix contained 2x Multiplex PCR Master Mix (Qiagen), the indicated standard and 42 primers at 1X nM

5   concentration labeled with different mass tags. Background in reactions without standard (no template control, 12.5 ng human DNA) was subtracted and the sum of Integrated Ion Current for both tags was plotted.

10   Figure 17: This figure shows analysis of clinical specimens; respiratory infection. RNA from clinical specimens was extracted by standard procedures and reverse transcribed into cDNA (Superscript RT system, Invitrogen, Carlsbad, CA; 20 ul volume). Five microliter

15   of reaction was then subjected to mass tag PCR.

Figure 18: This figure shows multiplex mass tag PCR analysis of six human respiratory specimens. Mass tag primer sets employed in a single tube assay are indicated

20   at the bottom of the figure.

Figure 19: This figure shows structures of MASSCODE tags.

Figure 20: This figure shows differential real-time PCR

25   for West Nile virus and St. Louis encephalitis virus.

Figures 21A-21B: (A) This figure shows serial dilutions of plasmid standards (5×10^5, 5×10^4, 5×10^3, 5×10^2, 5×10^1, and 5×10^0) for RSV group A, RSV group B,

30   Influenza A, HCoV-SARS, HCoV-229E, HCoV-OC43, and M. pneumoniae were each analyzed by mass tag PCR in a multiplex format. (B) This figure shows simultaneous

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detection of multiple targets in multiplex format using mixtures of two templates per assay (5×10^4 copies each): HCoV-SARS and M. pneumoniae, HCoV-229E and M. pneumoniae, HCoV-OC43 and M. pneumoniae, and HCoV-229E and HCoV-OC43.

5

Figure 22: This figure shows a schematic of the mass tag PCR procedure.

Figure 23: Thus figure shows identification of various
10 infections using masscode tags.

Detailed Description of the Invention

Terms

5

As used herein, and unless stated otherwise, each of the following terms shall have the definition set forth below.

10   "Mass tag" shall mean any chemical moiety (i) having a fixed mass, (ii) affixable to a nucleic acid, and (iii) whose mass is determinable using mass spectrometry. Mass tags include, for example, chemical moieties such as small organic molecules, and have masses which range, for 15   example, from 100Da to 2500Da.

"Nucleic acid" shall mean any nucleic acid molecule, including, without limitation, DNA, RNA and hybrids thereof. The nucleic acid bases that form nucleic acid 20   molecules can be the bases A, C, G, T and U, as well as derivatives thereof. Derivatives of these bases are well known in the art, and are exemplified in PCR Systems, Reagents and Consumables (Perkin Elmer Catalogue 1996-1997, Roche Molecular Systems, Inc., Branchburg, New 25   Jersey, USA).

"Pathogen" shall mean an organic entity including, without limitation, viruses and bacteria, known or suspected to be involved in the pathogenesis of a disease 30   state in an organism such as an animal or human.

"Sample" shall include, without limitation, a biological

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sample derived from an animal or a human, such as cerebro-spinal fluid, lymph, blood, blood derivatives (e.g. sera), liquidized tissue, urine and fecal material.

5 "Simultaneously detecting", with respect to the presence of target nucleic acids in a sample, means determining, in the same reaction vessels(s), whether none, some or all target nucleic acids are present in the sample. For example, in the instant method of simultaneously 10 detecting in a sample the presence of one or more of 50 target nucleic acids, the presence of each of the 50 target nucleic acids will be determined simultaneously, so that results of such detection could be, for example, (i) none of the target nucleic acids are present, (ii) 15 five of the target nucleic acids are present, or (iii) all 50 of the target nucleic acids are present.

"Specific", when used to describe a primer in relation to a target nucleic acid, shall mean that, under primer 20 extension-permitting conditions, the primer specifically binds to a portion of the target nucleic acid and is extended.

"Target nucleic acid" shall mean a nucleic acid whose 25 presence in a sample is to be detected by any of the instant methods.

"5-UTR" shall mean the 5'-end untranslated region of a nucleic that encodes a protein.
30
The following abbreviations shall have the meanings set forth below: "A" shall mean Adenine; "bp" shall mean base

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pairs; "C" shall mean Cytosine; "DNA" shall mean
deoxyribonucleic acid; "G" shall mean Guanine; "mRNA"
shall mean messenger ribonucleic acid; "RNA" shall mean
ribonucleic acid; "PCR" shall mean polymerase chain
5   reaction; "T" shall mean Thymine; "U" shall mean Uracil;
"Da" shall mean dalton.

Finally, with regard to the embodiments of this
invention, where a numerical range is stated, the range
10 is understood to encompass the embodiments of each and
every integer between the lower and upper numerical
limits. For example, the numerical range from 1 to 5 is
understood to include 1, 2, 3, 4, and 5.

15 <u>Embodiments of the Invention</u>

To address the need for enhanced multiplex capacity in
diagnostic molecular microbiology we have established a
PCR platform based on mass tag reporters that are easily
20 distinguished in Mass Spectrometry (MS) as discrete
signal peaks. Major advantages of the PCR/MS system
include: (1) hybridization to only two sites is required
(forward and reverse primer binding sites) vs real time
PCR where an intermediate third oligonucleotide is used
25 (probe binding site); this enhances flexibility in primer
design; (2) tried and proven consensus PCR primers can be
adapted to PCR/MS; this reduces the time and resources
that must be invested to create new reagents and assay
controls; (3) the large repertoire of tags allows highly
30 multiplexed assays; additional tags can be easily
synthesized to allow further complexity; and (4)
sensitivity of real time PCR is maintained. We view

PCR/MS as a tool with which to rapidly screen clinical materials for the presence of candidate pathogens. Thereafter, targeted secondary tests, including real time PCR, can be used to quantitate microbe burden and pursue
5   epidemiologic studies.

Specifically, this invention provides a method for simultaneously detecting in a sample the presence of one or more of a plurality of different target nucleic
10 acids comprising the steps of:

(a)   contacting the sample with a plurality of nucleic acid primers simultaneously and under conditions permitting, and for a time sufficient for, primer extension to occur, wherein (i) for each target
15   nucleic acid at least one predetermined primer is used which is specific for that target nucleic acid, (ii) each primer has a mass tag of predetermined size bound thereto via a labile bond, and (iii) the mass tag bound to any primer specific
20   for one target nucleic acid has a different mass than the mass tag bound to any primer specific for any other target nucleic acid;

(b)   separating any unextended primers from any extended primers;
25 (c)   simultaneously cleaving the mass tags from any extended primers; and

(d)   simultaneously determining the presence and sizes of any mass tags so cleaved,

wherein the presence of a cleaved mass tag having the
30 same size as a mass tag of predetermined size previously bound to a predetermined primer indicates the presence in the sample of the target nucleic acid specifically

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recognized by that predetermined primer.

In one embodiment of the instant method, the method
detects the presence in the sample of 10 or more
5   different target nucleic acids. In another embodiment,
the method detects the presence in the sample of 50 or
more different target nucleic acids. In a further
embodiment, the method detects the presence in the sample
of 100 or more different target nucleic acids. In a
10   further embodiment, the method detects the presence in
the sample of 200 or more different target nucleic acids.

In one embodiment of the instant method, the sample is
contacted with 4 or more different primers. In another
15   embodiment, the sample is contacted with 10 or more
different primers. In a further embodiment, the sample is
contacted with 50 or more different primers. In a further
embodiment, the sample is contacted with 100 or more
different primers. In yet a further embodiment, the
20   sample is contacted with 200 or more different primers.

In one embodiment of the instant method, one or more
primers comprises the sequence set forth in one of SEQ ID
NOs:1-96, and 98-101.
25
In another embodiment of the instant method, at least two
different primers are specific for the same target
nucleic acid. For example, in one embodiment a first
primer is a forward primer for the target nucleic acid
30   and a second primer is a reverse primer for the same
target nucleic acid. In this example, the mass tags bound
to the first and second primers can be of the same size

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or of different sizes. In another embodiment, a first
primer is directed to a 5'-UTR of the target nucleic acid
and a second primer is directed to a 3D polymerase region
of the target nucleic acid.

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In one embodiment of the instant method, wherein each
primer is from 15 to 30 nucleotides in length. In another
embodiment, each mass tag has a molecular weight of from
100Da to 2,500Da. In a further embodiment, the labile
10   bond is a photolabile bond, such as a photolabile bond
cleavable by ultraviolet light.

In another embodiment of the instant method, at least one
target nucleic acid is from a pathogen. Pathogens
15 . include, without limitation, B. anthracis, a Dengue
virus, a West Nile virus, Japanese encephalitis virus,
St. Louis encephalitis virus, Yellow Fever virus, La
Crosse virus, California encephalitis virus, Rift Valley
Fever virus, CCHF virus, VEE virus, EEE virus, WEE virus,
20   Ebola virus, Marburg virus, LCMV, Junin virus, Machupo
virus, Variola virus, SARS corona virus, an enterovirus,
an influenza virus, a parainfluenza virus, a respiratory
syncytial virus, a bunyavirus, a flavivirus, and an
alphavirus.

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In another embodiment, the pathogen is a respiratory
pathogen. Respiratory pathogens include, for example,
respiratory syncytial virus A, respiratory syncytial
virus B, Influenza A (N1), Influenza A (N2), Influenza A
30 . (M), Influenza A (H1), Influenza A (H2), Influenza A
(H3), Influenza A (H5), Influenza B, SARS coronavirus,
229E coronavirus, OC43 coronavirus, Metapneumovirus

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European, Metapneumovirus Canadian, Parainfluenza 1, Parainfluenza 2, Parainfluenza 3, Parainfluenza 4A, Parainfluenza 4B, Cytomegalovirus, Measles virus, Adenovirus, Enterovirus, M. pneumoniae, L. pneumophilae,

5   and C. pneumoniae.

In a further embodiment, the pathogen is an encephalitis-inducing pathogen. Encephalitis-inducing pathogens include, for example, West Nile virus, St. Louis

10   encephalitis virus, Herpes Simplex virus, HIV 1, HIV 2, N. meningitides, S. pneumoniae, H. influenzae, Influenza B, SARS coronavirus, 229E-CoV, OC43-CoV, Cytomegalovirus, and a Varicella Zoster virus. In a further embodiment, the pathogen is a hemorrhagic fever-inducing pathogen. In

15   a further embodiment, the sample is a forensic sample, a food sample, blood, or a derivative of blood, a biological warfare agent or a suspected biological warfare agent.

20   In one embodiment of the instant method, the mass tag is selected from the group consisting of structures V1 to V4 of Fig. 1 or Fig. 8.

In another embodiment of the instant method, the presence

25   and size of any cleaved mass tag is determined by mass spectrometry. Mass spectrometry includes, for example, atmospheric pressure chemical ionization mass spectrometry, electrospray ionization mass spectrometry, and matrix assisted laser desorption ionization mass

30   spectrometry.

In one embodiment of the instant method, the target

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nucleic acid is a ribonucleic acid. In another
embodiment, the target nucleic acid is a deoxyribonucleic
acid. In a further embodiment, the target nucleic acid is
from a viral source.

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This invention provides a kit for simultaneously
detecting in a sample the presence of one or more of a
plurality of different target nucleic acids comprising a
plurality of nucleic acid primers wherein (i) for each

10   target nucleic acid at least one predetermined primer is
used which is specific for that target nucleic acid, (ii)
each primer has a mass tag of predetermined size bound
thereto via a labile bond, and (iii) the mass tag bound
to any primer specific for one target nucleic acid has a

15   different mass than the mass tag bound to any primer
specific for any other target nucleic acid.

This invention also provides a kit for simultaneously
detecting in a sample the presence of one or more of a

20   plurality of different target nucleic acids comprising
(a) a plurality of nucleic acid primers wherein (i) for
each target nucleic acid at least one predetermined
primer is used which is specific for that target nucleic
acid, (ii) each primer has a mass tag of predetermined

25   size bound thereto via a labile bond, and (iii) the mass
tag bound to any primer specific for one target nucleic
acid has a different mass than the mass tag bound to any
primer specific for any other target nucleic acid; and
(b) a mass spectrometer.

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This invention further provides a kit for simultaneously
detecting in a sample the presence of one or more of a

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plurality of different target nucleic acids comprising
(a) a plurality of nucleic acid primers wherein (i) for
each target nucleic acid at least one predetermined
primer is used which is specific for that target nucleic

5   acid, (ii) each primer has a mass tag of predetermined
size bound thereto via a labile bond, and (iii) the mass
tag bound to any primer specific for one target nucleic
acid has a different mass than the mass tag bound to any
primer specific for any other target nucleic acid, and

10   (b) instructions for use.

Finally, this invention provides a kit for simultaneously
detecting in a sample the presence of one or more of a
plurality of different target nucleic acids comprising

15   (a) a plurality of nucleic acid primers wherein (i) for
each target nucleic acid at least one predetermined
primer is used which is specific for that target nucleic
acid, (ii) each primer has a mass tag of predetermined
size bound thereto via a labile bond, and (iii) the mass

20   tag bound to any primer specific for one target nucleic
acid has a different mass than the mass tag bound to any
primer specific for any other target nucleic acid; (b) a
mass   spectrometer;   and   (c)   instructions   for
simultaneously detecting in a sample the presence of one

25   or more of a plurality of different target nucleic acids
using the primers and the mass spectrometer.

This invention will be better understood by reference to
the Experimental Details which follow, but those skilled

30   in the art will readily appreciate that the specific
experiments detailed are only illustrative of the
invention as described more fully in the claims which

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follow thereafter.

Experimental Details

5   <u>**Example 1**</u>

Abbreviations: 5'-UTR, 5'-untranslated region; ALS, Amyotrophic Lateral Sclerosis; APCI, atmospheric pressure chemical ionization; ESI, electrospray ionization; PCR,
10   polymerase chain reaction; MALDI-TOF, matrix assisted laser desorption ionization time of flight; MS, mass spectrometry

Background

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Establishing a causal relationship between infection with a virus and a specific disease may be complex. In most acute viral diseases, the responsible agent is readily implicated because it replicates at high levels in the
20   affected tissue at the time the disease is manifest, morphological changes consistent with infection are evident, and the agent is readily cultured with standard microbiological techniques. In contrast, implication of viruses in chronic diseases may be confounded because
25   persistence requires restricted gene expression, classical hallmarks of infection are absent, and/or mechanisms of pathogenesis are indirect or subtle. Methods for cloning nucleic acids of microbial pathogens directly from clinical specimens offer new opportunities
30   to investigate microbial associations in chronic diseases (21). The power of these methods is that they can succeed where methods for pathogen identification through

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serology or cultivation may fail due to absence of
specific reagents or fastidious requirements for agent
replication. Over the past decade, the application of
molecular pathogen discovery methods resulted in
5   identification of novel agents associated with both acute
and chronic diseases, including Borna disease virus,
Hepatitis C virus, Sin Nombre virus, HHV-6, HHV-8,
Bartonella henselae, and *Tropherema whippeli* (5-7, 17,
19, 22, 23, 27).

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Various methods are employed or proposed for cultivation-
independent characterization of infectious agents. These
can be broadly segregated into methods based on direct
analysis of microbial nucleic acid sequences (e.g., cDNA
15   microarrays, consensus PCR, representational difference
analysis, differential display), direct analysis of
microbial protein sequences (e.g., mass spectrometry),
immunological systems for microbe detection (e.g.,
expression libraries, phage display) and host response
20   profiling. A comprehensive program in pathogen discovery
will need to exploit most, if not all, of these
technologies.

The decision to employ a specific method is guided by the
25   clinical features, epidemiology, and spectrum of
potential pathogens to be implicated. Expression
libraries, comprised of cDNAs or synthetic peptides, may
be useful tools in the event that large quantities of
acute and convalescent sera or cerebrospinal fluid are
30   available for screening purposes; however, the approach
is cumbersome, labor-intensive, and success is dependent
on the presence of a specific, high affinity humoral

immune response. The utility of host response mRNA profile analysis has been demonstrated in several *in vitro* paradigms and some inbred animal models (8, 26, 30); nonetheless, it is important to formally consider

5   the possibility that a variety of organisms may activate similar cascades of chemokines, cytokines, and other soluble factors that influence host gene expression to produce what are likely to be convergent gene expression profiles. Thus, at least in virology, it is prudent to

10 explore complementary methods for pathogen identification based on agent-encoded nucleic acid motifs. Given the potential for high density printing of microarrays, it is feasible to design slides or chips decorated with both host and pathogen targets. This would provide an

15 unprecedented opportunity to simultaneously survey host response mRNA profiles and viral flora, providing insights into microbial pathogenesis not apparent with either method of analysis alone. Representational difference analysis (RDA) is an important tool for

20 pathogen identification and discovery. However, RDA is a subtractive cloning method for binary comparisons of nucleic acid populations (12, 18). Thus, although ideal for analysis of cloned cells or tissue samples that differ only in a single variable of interest, RDA is less

25 well suited to investigation of syndromes wherein infection with any of several different pathogens results in similar clinical manifestations, or infection is not invariably associated with disease. An additional caveat is that because the method is dependent upon the presence

30 of a limited number of restriction sites, RDA is most likely to succeed for agents with large genomes. Indeed, in this context, it is noteworthy that the two viruses

24

detected by RDA in the listing above (see first paragraph) were herpesviruses (5, 6). Consensus PCR (cPCR) has been a remarkably productive tool for biology. In addition to identifying pathogens, particularly

5   genomes of prokaryotic pathogens, this method has facilitated identification of a wide variety of host molecules, including cytokines, ion channels, and receptors. Nonetheless, until recently, a difficulty in applying cPCR to pathogen discovery in virology has been

10   that it is difficult to identify conserved viral sequences of sufficient length to allow cross-hybridization, amplification, and discrimination using traditional cPCR format. While this may not be problematic when one is targeting only a single virus

15   family, the number of assays required becomes infeasible when preliminary data are insufficient to allow a directed, limited analysis. To address this issue, we adapted cPCR to Differential Display, a PCR-based method for simultaneously displaying the genetic composition of

20   multiple sample populations in an acrylamide gel format (16). This hybrid method, domain-specific differential display (DSDD), employs short, degenerate primer sets designed to hybridize to viral genes representing larger taxonomic categories than can be resolved in cPCR. The

25   major advantages to this approach are: (i) reduction in numbers of reactions required to identify genomes of known viruses, and (ii) potential to detect viruses less closely related to known viruses than those found through cPCR. The differential display format also permits

30   identification of syndrome-specific patterns of gene expression (host and pathogen) that need not be present in all clinical samples. Additionally, because multiple

25

samples can be analyzed in side-by-side comparisons, DSDD allows examination of the timecourse of gene expression patterns. Lastly, recent experience with isolation of the West Nile virus responsible for the outbreak of

5   encephalitis in New York in the summer of 1999 indicates that DSDD may be advantageous in instances where template is suboptimal due to degradation (e.g., postmortem field specimens).

10   The development and application of sensitive high throughput methods for detecting a wide range of viruses is anticipated to provide new insights into the pathogenesis of chronic diseases. We are funded through AI51292 to support these objectives by establishing DNA

15   microarray, multiplexed bead-based flow cytometric (MB-BFC) and domain specific differential display (DSDD) assay platforms for viral surveillance and discovery in chronic diseases. Each of these methods has its strengths; however, none is ideal. Microarrays provide a

20   platform wherein one can simultaneously query thousands of microbial and host gene targets but lack sensitivity and are difficult to modify as new targets are identified. Bead-based arrays are flexible but similar in sensitivity to microarrays.

25

Domain specific differential display is sensitive and flexible but labor intensive. Real time PCR (not a component of our original application but useful to note for purposes of method comparisons), is rapid and

30   sensitive, but cannot be used for broad range detection of viral sequences, because of stringent sequence constraints for the three oligonucleotides comprising the

system (two primers, one probe).

Mass-Tag PCR would integrate PCR and mass spectrometry (MS) into a stable and sensitive digital assay platform.

5   It is similar in sensitivity and efficiency to real time PCR but provides the advantages of simultaneous detection and discrimination of multiple targets, due to less stringent constraints on primer selection. Additionally, whereas multiplexing is limited in real time PCR by

10 overlapping fluorescence emission spectra, Mass-Tag PCR allows discrimination of a large repertoire of mass tags with molecular weights between 150 and 2500 daltons.

In Mass-Tag PCR, virus identity is be defined by the

15 presence of label of a specific molecular weight associated with an amplification product. Primers are be designed such that the tag can be cleaved by irradiation with UV light. Following PCR, the amplification product can be immobilized on a solid support and excess soluble

20 primer removed. After cleavage by UV irradiation (~350 nm), the released tag will be analyzed by mass spectrometry. Detection is sensitive, fast, independent of DNA fragment length, and ideally suited to the multiplex format required to survey clinical materials

25 for infection with a wide range of infectious agents.

Results

Mass spectrometry (MS) is a rapid, sensitive method for

30 detection of small molecules. With the development of new ionization techniques such as matrix assisted laser desorption ionization (MALDI) and electrospray ionization

(ESI), mass spectrometry has become an indispensable tool in many areas of biomedical research. Although these ionization methods are suitable for the analysis of bioorganic molecules, such as peptides and proteins,

5   improvements in both detection and sample preparation will be required before mass spectrometry can be used to directly detect long DNA fragments. A major confound in exploiting MS for genetic investigation has been that long DNA molecules are fragmented during the analytic

10   process. The mass tag approach overcomes this limitation by detecting small stable mass tags that serve as signatures for specific DNA sequences rather than the DNA sequences themselves.

15   Atmospheric pressure chemical ionization (APCI) has advantages over ESI and MALDI for some applications. Because buffer and inorganic salts impact ionization efficiency, performance in ESI is critically dependent upon sample preparation conditions. In MALDI, matrix must

20   be added prior to sample introduction into the mass spectrometer; speed is often limited by the need to search for an ideal irradiation spot to obtain interpretable mass spectra. APCI requires neither desalting nor mixing with matrix to prepare crystals on a

25   target plate. Therefore in APCI, mass tag solutions can be injected directly. Because mass tags are volatile and have small mass values, they are easily detected by APCI ionization with high sensitivity. The APCI mass tag system is easily scaled up for high throughput operation.

30

We have established methods for synthesis and APCI analysis of mass tags coupled to DNA fragments.

Precursors of four mass tags [(a) acetophenone; (b) 3-fluoroacetophenone; (c) 3,4-difluoroacetophenone; and (d) 3,4-dimethoxyacetophenone] are shown in Fig. 1. Upon nitration and reduction, the photoactive tags are

5   produced and used to code for the identity of up to four different primer pairs (or target sequences). In a simulation experiment, we have obtained clean APCI mass spectra for the 4 mass tag precursors (a, b, c, d) as shown in Fig. 2. The peak with m/z of 121 is a, 139 is b,

10   157 is c and 181 is d. This result indicates that the 4 compounds we designed as mass tags are stable and produce discrete high resolution digital data in an APCI mass spectrometer. In the research described below, each of the unique m/z from each mass tag translates to the

15   identity of a viral sequence (V) [Tag-1 (m/z,150) = V-1; Tag-2 (m/z,168) = V-2; Tag-3 (m/z,186) = V-3; Tag-4 (m/z,210) = V-4]. A variety of functional groups can be introduced to the mass tag parent structure for generating a large number of mass tags with different

20   molecular weights. Thus, a library of primers labeled with mass tags that can discriminate between hundreds of viral sequence targets.

DNA sequencing with biotinylated dideoxynucleotides on a
25   *mass spectrometer*

PCR amplification can be nonspecific; thus, products are commonly sequenced to verify their identity as bona fide targets. Here we apply the rapidity and sensitivity of
30   mass tag analyses to direct MS-sequencing of PCR amplified transcripts.

MALDI-TOF MS has recently been explored widely for DNA sequencing. The Sanger dideoxy procedure (25) is used to generate the DNA sequencing fragments. The mass resolution in theory can be as good as one dalton;

5   however, in order to obtain accurate measurement of the mass of the sequencing DNA fragments, the samples must be free from alkaline and alkaline earth salts and falsely stopped DNA fragments (fragments terminated at dNTPs instead of ddNTPs). Our method for preparing DNA

10   sequencing fragments using biotinylated dideoxynucleotides and a streptavidin-coated solid phase is shown in Fig. 3. DNA template, dNTPs (A, C, G, T) and ddNTP-biotin (A-b, C-b, G-b, T-b), primer and DNA polymerase are combined in one tube. After polymerase

15   extension and termination reactions, a series of DNA fragments with different lengths are generated. The sequencing reaction mixture is then incubated for a few minutes with a streptavidin-coated solid phase. Only the DNA sequencing fragments that are terminated with

20   biotinylated dideoxynucleotides at the 3' end are captured on the solid phase. Excess primers, falsely terminated DNA fragments, enzymes and all other components from the sequencing reaction are washed away. The biotinylated DNA sequencing fragments are then

25   cleaved off the solid phase by disrupting the interaction between biotin and streptavidin using ammonium hydroxide or formamide to obtain a pure set of DNA sequencing fragments. These fragments are then mixed with matrix (3-hydroxypicolinic acid) and loaded onto a mass

30   spectrometer to produce accurate mass spectra of the DNA sequencing fragments. Since each type of nucleotide has a unique molecular mass, the mass difference between

adjacent peaks of the mass spectra gives the sequence identity of the nucleotides. In DNA sequencing with mass spectrometry, the purity of the samples directly affects the quality of the obtained spectra. Excess primers,

5   salts, and fragments that are prematurely terminated in the sequencing reactions (false stops) will create extra noise and extraneous peaks (11). Excess primers can also dimerize to form high molecular weight species that give a false signal in mass spectrometry (29). False stops

10 occur in DNA sequencing reaction when a deoxynucleotide rather than a dideoxynucleotide terminates a sequencing fragment. A deoxynucleotide terminated false stop has a mass difference of 16 daltons compared with its dideoxy counterpart. This mass difference is identical to the

15 difference between adenine and guanine. Thus, false stops can be misinterpreted or interfere with existing peaks in the mass spectra. Our method is designed to eliminate these confounds. We previously established a procedure for accurately sequencing DNA using fluorescent dye-

20 labeled primers and biotinylated dideoxynucleotides. In this procedure, accurate and clean DNA sequencing data were obtained by removing falsely stopped fragments prior to analysis through use of an intermediate purification step on streptavidin-coated magnetic beads (13, 14).

25

Sequencing experiments for a 55 bp synthetic template using MALDI-TOF mass spectrometry were recently performed (9). Four commercially available biotinylated dideoxynucleotides   ddATP-11-biotin,   ddGTP-11-biotin,

30 ddCTP-11-biotin and ddTTP-11-biotin (NEN, Boston) were used to produce the sequencing ladder in a single tube by cycle sequencing. Clean sequence peaks were obtained on

31

the mass spectra, with the first peak being primer
extended by one biotinylated dideoxynucleotide. Although
the identity of A and G residues were determined
unambiguously, C and T could not be differentiated
5   because the one dalton mass difference between the ddCTP-
11-biotin and ddTTP-11-biotin cannot be consistently
resolved by using the current mass detector for DNA
fragments.   Nonetheless, these results confirmed that
clean   sequencing   ladders   can   be   obtained   by
10   capture/release of DNA sequencing fragments with biotin
located on the 3' dideoxy terminators. The procedure has
been improved by using biotinylated ddTTPs that have
large mass differences in comparison to ddCTP-11-biotin.
Pairing ddTTP-16-biotin (Enzo, Boston), which has a large
15   mass difference in comparison to ddCTP-11-biotin, with
ddATP-11-biotin,   ddCTP-11-biotin,   and   ddGTP-11-biotin,
allowed unambiguous sequence determination in the mass
spectra (Fig. 4). Mass spectrum from Sanger sequencing
reactions   using   dd(A,G,C)TP-11-biotin   and   ddTTP-16-
20   biotin. All four bases are unambiguously identified in
the spectrum. Data presented here were generated using a
synthetic template mimicking a portion of the HIV type 1
protease gene.   DNA sequencing was performed in one tube
by combining the biotinylated ddNTPs, regular dNTPs, DNA
25   polymerase, and reaction buffer (9).

Table 1

Cloned enterovirus targets		
Virus	5' UTR	pol
Echovirus 3	+	+
Echovirus 6	+	+
Echovirus 9	+	+
Echovirus 16	+	+
Echovirus 17	+	+
Echovirus 25	+	+
Echovirus 30	+	+
Poliovirus 1	+	+
Poliovirus 2	+	+
Poliovirus 3	+	+
Coxsackie A9	+	+
Coxsackie B2	+	+

In Propagation

Coxsackie (A9), Coxsack ie A16, Coxsacki e
B1, Coxsacki e B3, Coxsacki e B4, Coxsacki e
B5, Coxsacki e B6, Echovirus 7, Echovirus
13, Echovirus 18

Cloning viral targets as controls for Mass-Tag PCR

Multiple sequence alignment algorithms have been used by
5   our bioinformatics core to extract the most conserved
genomic regions amongst the GenBank published enteroviral
sequences. Regions wherein sequence conservation meets or
exceeds 80% for an enteroviral serogroup or genetically
related subgroup have been identified in the 5'-
10 untranslated region (UTR) and the polymerase gene (3D) of
the enterovirus genus. A representative collection of
virus isolates has been obtained to generate calibrated
standards for Mass-Tag PCR (Table 1). The current panel
includes 22 isolates representing all characterized
15 serogroups of pathogenic relevance (A, B, C, and D;
covering about 90% of all US enterovirus isolates in the
past 10 years; the remaining 10% include non-typed
isolates). Twelve isolates have been grown and the
relevant regions cloned for spotting onto DNA microarrays
20 and use as transcript controls for DSDD, multiplex bead
based, and real time PCR assays. Viruses can be
propagated in the appropriate cell lines to generate
working and library stocks (Rd, Vero, HeLa, Fibroblast,
or WI-38 cells). Library stocks can be frozen and
25 maintained in curated collections at -70°C. Viral RNA
can be extracted from working stocks using Tri-Reagent
(Molecular Research Center, Inc.). Purified RNA can be
reverse transcribed into cDNA using random hexamer
priming [to avoid 3' bias] (Superscript II,
30 Invitrogen/Life Technologies).

Target regions of 100-200 bp representing the identified

34

core sequences will be amplified by PCR from cDNA template using virus-specific primers. Products are cloned (via a single deoxyadenosine residue added in template-independent fashion by common Taq-polymerases to

5 3'-ends of amplification products) into the transcription vector pGEM T-Easy (Promega Corp.). After transformation and amplification in *Escherichia coli*, plasmids are analyzed by restriction mapping and automated dideoxy sequencing (Columbia Genome Center) to determine insert

10 orientation and fidelity of PCR. Plasmid libraries will be maintained as both cDNAs and glycerol stocks.

Multiple sequence alignment algorithms can be used to identify highly conserved (>95%) sequence stretches of

15 20-30 bp length within the identified core sequences to serve as targets for primer design.

Synthesis of Primers for Use in Mass-Tag PCR

20 Highly conserved target regions within the core sequences suitable for primer design are identified by using multiple sequence alignment algorithms adjusted for the appropriate window size (20-30 bp) and conservation threshold (>95%). Final alignments are color-coded to

25 facilitate manual inspection. Parameters implicated in primer performance including melting temperature, 3'-terminal stability, internal stability, and propensity of potential primers to form stem loops or primer-dimers can be assessed using standard primer selection software

30 programs OLIGO (Molecular Biology Insights), Primer Express (PE Applied Biosystems), and Primer Premiere (Premiere Biosoft International). Primers can be

synthesized with a primary amine-group at the 5'-end for subsequent coupling to NHS esters of the mass tags (Fig. 5). Mass tags with molecular weights between 150 and 2500 daltons can be generated by introducing various

5　functional groups [Rn] in the mass tag parent structure to code for individual primers and thus for the targeted viral sequence (see Fig. 6; also showing the photocleavage reaction). MS is capable of detecting small stable molecules with high sensitivity, a mass resolution

10　greater than one dalton, and the detection requires only microseconds. The mass tagging approach has been successfully used to detect multiplex single nucleotide polymorphisms (15).

15　*Sensitivity and Specificity of Mass-Tag PCR for Detection of Enteroviral Transcripts*

Although the method disclosed here is useful for detecting viral RNA, plasmid DNA is an inexpensive,

20　easily quantitated sequence target; thus, primer sets can be initially validated by using dilutions of linearized plasmid DNA. Plasmids are selected to carry the viral insert in mRNA sense orientation with respect to the T7 promoter sequence. Plasmids will be linearized by

25　restriction digestion using an appropriate enzyme that cleaves in the polylinker region downstream of the insert. Where the cloned target sequence is predicted to contain the available restriction sites, a suitable unique restriction site is introduced via the PCR primer

30　used during cloning of the respective target. Purified linearized plasmid DNA is serially diluted in background DNA (human placenta DNA, Sigma) to result in 5×10^5, $5 \times$

10^4, 5 x 10^3, 5 x 10^2, 5 x 10^1, and 5 x 10^0 copies per assay.

Once optimal primer sets for detection of all relevant
5   enteroviruses are identified, the sensitivity of the
entire procedure including RNA extraction and reverse
transcription is assessed. Synthetic RNA transcripts of
each target sequence are generated from the linearized
plasmid DNA using T7 RNA polymerase. Transcripts are
10   serially diluted in background RNA relevant to the
primary hypothesis (e.g., ALS, normal spinal cord RNA).
Individual dilutions representing 5 x 10^5, 5 x 10^4, 5 x
10^3, 5 x 10^2, 5 x 10^1, and 5 x 10^0 copies per assay in a
background of 25 ng/ul total RNA are extracted with Tri-
15   Reagent, reverse transcribed, and then subjected to Mass-
Tag PCR.

Specificity of the identified primer sets relevant to
multiplexing can be assessed by using one desired primer
20   set in conjunction with its respective target sequence at
5 times threshold concentration in the presence of all
other, potentially cross-reacting, target sequences at a
10^2-, 10^4- and 10^6-fold excess.

25   PCR amplification is performed using photocleavable mass
tagged primers in the presence of a biotinylated
nucleotide (e.g. Biotin-16-dUTP, Roche) to allow removal
of excess primer after PCR. Amplification products will
be purified from excess primer by binding to a
30   streptavidin-coated solid phase such as streptavidin-
Sepharose (Pharmacia) or streptavidin coated magnetic
beads (Dynal) via biotin-streptavidin interaction.

Molecular mass tags can be made cleavable by irradiation with near UV light (~350 nm), and the released tags introduced by either chromatography or flow injection into a pneumatic nebulizer for detection in an

5   atmospheric pressure chemical ionization mass spectrometer. Alternatively, to increase the specificity of detection by analyzing only PCR products of the expected size range, the mass tagged amplicons, can be size-selected (without the requirement for biotinylated

10   nucleotides) using HPLC.

Multiplex Detection and Identification of Enteroviral Transcripts

15   A method that allows simultaneous detection of a broad range of enteroviruses with similar sensitivity was developed. A series of 4 primer sets were identified in the 5'-UTR predicted to detect all enteroviruses. These can be combined into two or perhaps even one mixed set

20   for multiplex PCR. Two different genomic regions, 5'-UTR and polymerase, are targeted with independent primer panels, in order to confirm presence of enterovirus infection.

25   Once the presence of enteroviral sequences are confirmed using broad range primer sets, a different primer set is used to discriminate amongst the various enteroviral species. Whereas broad range primers are be selected from the highly conserved 5'-UTR and polymerase 3D gene

30   regions, the primer sets used to identify the enterovirus species target the most divergent genomic regions in VP3 and VP1.

Limitations must be considered in that although cerebral
spinal fluid is unlikely to contain more than a single
enterovirus (the virus responsible for clinical disease
5   in an individual patient), individual stool samples may
contain several enteroviruses. It is important,
therefore, that assays not favor amplification or
detection of one viral species over another. Second,
multiplexing can result in loss of sensitivity. Thus,
10   panels should be assessed for sensitivity (and
specificity) with addition of new primer sets.

*Direct MS-sequencing of PCR Amplified Enteroviral
Transcripts for virus species identification*

15

MALDI MS has been explored widely for DNA sequencing;
however, this approach requires that the DNA sequencing
fragments be free from alkaline and alkaline earth salts,
as well as other contaminants, to ensure accurate
20   measurements of the masses of the DNA fragments. We
explored a novel MS DNA sequencing method that generates
Sanger-sequencing   fragments   using   biotinylated
dideoxynucleotides labeled with mass tags.

25   The ability to distinguish various nucleotide bases in
DNA using mass spectrometry is dependent on the mass
differences of the DNA ladders in the mass spectra.
Smith et al. have shown that using dye labeled ddNTP
paired with a regular dNTP to space out the mass
30   difference can increase the detection resolution in a
single nucleotide extension assay (10). Preliminary
studies using biotin-11-dd(A, C, G)TPs and biotin-16-

ddTTP, indicated that the smallest mass difference between any two nucleotides is 16 daltons. To enhance the ability to distinguish peaks in the sequencing spectra, the mass separation of the individual ddNTPs can be

5   increased by systematically modifying the biotinylated dideoxynucleotides by incorporating mass linkers assembled using 4-aminomethyl benzoic acid derivatives. The mass linkers can be modified by incorporating one or two fluorine atoms to further space out the mass

10 differences between the nucleotides. The structures of the newly designed biotinylated ddNTPs are shown in Fig. 7. Linkers are attached to the 5 position on the pyrimidine bases (C and T), and to the 7 position on the purines (A and G) to facilitate conjugation with biotin.

15 It has been established that modification of these positions on the bases in the nucleotides, even with bulky energy transfer (ET) fluorescent dyes, still allows efficient incorporation of the modified nucleotides into the DNA strand by DNA polymerase (24, 31). Biotin and

20 the mass linkers are considerably smaller than the ET dyes, ameliorating difficulties in incorporation of ddNTP-linker-biotin molecules into DNA strands in sequencing reactions.

25 The DNA sequencing fragments that carry a biotin at the 3'-end are made free from salts and other components in the sequencing reaction by capture with streptavidin-coated magnetic beads. Thereafter, the correctly terminated biotinylated DNA fragments are released and

30 loaded onto the mass spectrometer. Results indicate that MS can produce high resolution of DNA-sequencing fragments, fast separation on microsecond time scales,

and eliminate the compressions associated with gel
electrophoresis.

Amplification products obtained by PCR with broad range
5   5'-UTR or polymerase 3D primer sets can be used as
template. Sequencing permits discrimination between bona
fide enteroviral amplification products and artifacts.
Where analysis of the semi-divergent sequence region
located toward the 3'-end of the 5'-UTR region is
10 inadequate for speciation, targeting the more divergent
VP3 and/or VP1 regions is preferred.

References for Example 1

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Example 2

Multiplex Mass Tag PCR Detection of Respiratory Pathogens

Background and Significance

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The advent of SARS in 2003 poignantly demonstrated the urgency of establishing rapid, sensitive, specific, inexpensive tools for differential laboratory diagnosis of infectious diseases. Through unprecedented global
10 collaborative efforts, the causative agent was rapidly implicated and characterized, facilitating development of serologic and molecular assays for infection, and containment of the outbreak. Nonetheless, as the northern hemisphere entered the winter season of 2004, the
15 diagnosis of SARS still rested on clinical and epidemiological as well as laboratory criteria.

Methods for cloning nucleic acids of microbial pathogens directly from clinical specimens offer new opportunities
20 to investigate microbial associations in diseases. The power of these methods is not only sensitivity and speed but also the potential to succeed where methods for pathogen identification through serology or cultivation may fail due to absence of specific reagents or
25 fastidious requirements for agent replication.

Various methods are employed or proposed for cultivation-independent characterization of infectious agents. These can be broadly segregated into methods based on direct
30 analysis of microbial nucleic acid sequences, direct analysis of microbial protein sequences, immunological

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systems for microbe detection, and host response profiling. Any comprehensive armamentarium should include most, if not all, of these tools. Nonetheless, classical methods for microbiology remain important. Indeed, the

5   critical breakthrough during the SARS outbreak was the cultivation of the agent in tissue culture.

Real-time PCR methods have significantly changed diagnostic molecular microbiology by providing rapid,

10   sensitive, specific tools for detecting and quantitating genetic targets. Because closed systems are employed, real-time PCR is less likely than nested PCR to be confounded by assay contamination due to inadvertent aerosol introduction of amplicon/positive control/cDNA

15   templates that can accumulate in diagnostic laboratories. The specificity of real time PCR is both a strength and a limitation. Although the potential for false positive signal is low so is the utility of the method for screening to detect related but not identical genetic

20   targets. Specificity in real-time PCR is provided by two primers (each approximately 20 matching nucleotides (nt) in length) combined with a specific reporter probe of about 27 nt. The constraints of achieving hybridization at all three sites may confound detection of diverse,

25   rapidly evolving microbial genomes such as those of single-stranded RNA viruses. These constraints can be compensated in part by increasing numbers of primer sets accommodating various templates. However, because real-time PCR relies on fluorescent reporter dyes, the

30   capacity for multiplexing is limited to the number of emission peaks that can be unequivocally separated. At present up to four dyes can be identified simultaneously.

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Although the repertoire may increase, it will unlikely to change dramatically.

To address the need for enhanced multiplex capacity in
5   diagnostic molecular microbiology we have established a
PCR platform based on mass tag reporters that are easily
distinguished in MS as discrete signal peaks. Major
advantages of the PCR/MS system include: (1)
hybridization to only two sites is required (forward and
10   reverse primer binding sites) vs real time PCR where an
intermediate third oligonucleotide is used (probe binding
site); this enhances flexibility in primer design; (2)
tried and proven consensus PCR primers can be adapted to
PCR/MS; this reduces the time and resources that must be
15   invested to create new reagents and assay controls; (3)
the large repertoire of tags allows highly multiplexed
assays; additional tags can be easily synthesized to
allow further complexity; and (4) sensitivity of real
time PCR is maintained. We view PCR/MS as a tool with
20 · which to rapidly screen clinical materials for the
presence of candidate pathogens. Thereafter, targeted
secondary tests, including real time PCR, can be used to
quantitate microbe burden and pursue epidemiologic
studies.
25

Preliminary Data

We have developed bioinformatic tools to facilitate
sequence alignments, motif identification, and primer
30   design; established banks of viral strains, cDNA
templates, and primers; and built relationships with
collaborators in national and global public health

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laboratory networks that provide access to data, organisms, sera, and cDNAs that facilitate assay development and validation. Over the past two years we have integrated PCR and MS into a stable and sensitive

5   digital assay platform similar in sensitivity and efficiency to real time PCR but with the advantages of simultaneous detection and discrimination of multiple targets. Using the 4 tags created for DNA sequencing we initially tested the method with flavivirus and

10   bunyavirus targets as a proof of principle for an encephalitis project. The collaboration was later expanded to include two industrial partners: QIAGEN GmbH, a partner with a large validated library of proprietary photocleavable mass tags (Masscode™) and expertise in

15   manufacture and commercial distribution, and Griffin Analytical Technologies, a partner actively engaged in design and fabrication of low cost portable MS instruments for field applications.

20   *Selection of APCI LCMS Platform*

Mass spectrometry is a rapid, sensitive method for detection of small molecules. With the development of Ionization techniques such as matrix assisted laser

25   desorption ionization (MALDI) and electrospray ionization (ESI), MS has become a indispensable tool in many areas of biomedical research. Although these ionization methods are suitable for the analysis of bioorganic molecules, such as peptides and proteins, improvements in both

30   detection and sample preparation will be required before mass spectrometry can be used to directly detect long DNA fragments. A major confound in exploiting MS for genetic

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investigation has been that long DNA molecules are fragmented during the analytic process. The mass tag approach we have developed overcomes this limitation by detecting small stable mass tags that serve as signatures

5   for specific DNA sequences rather than the DNA sequences themselves.

We have explored the kinetics of photocleavable primer conjugation. Ionization and detection of the photocleaved

10   mass tags have been extensively characterized using atmospheric pressure chemical ionization (APCI) as the ionization source while using a single quadrupole mass spectrometer as the detector (Jingyue et al. , Kim et al. 2003; Kokoris et al. 2000). Because buffer and inorganic

15   salts impact ionization efficiency, performance in ESI was determined to be critically dependent upon sample preparation conditions. In MALDI, matrix must be added prior to sample introduction into the mass spectrometer, which is a time consuming step that requires costly

20   sample spotting instrumentation. Similary, speed is often limited by the need to search for an ideal irradiation spot to obtain interpretable mass spectra.

In contrast, APCI is much more tolerant of residual

25   inorganic salts (than ESI) and does not require mixing with matrix to prepare crystals on a target plate. Thus, mass tag solutions can be injected directly into the MS via a Liquid Chromatography (LC) delivery system. Since mass tags ionize well under APCI conditions and have

30   small mass values (less that 800 amu), they are detected with high sensitivity (< 5 femtomolar limit of detection) with the APCI-Quadrupole LCMS platform.

Methods for synthesis and APCI-MS analysis of mass tags coupled to DNA fragments are illustrated in Fig. 8 where precursors are (a) acetophenone; (b) 4-

5   fluoroacetophenone; (c) 3-methoxyacetophenone; and (d) 3,4-dimethoxyacetophenone.

Upon nitration and reduction, the photoactive tags are produced and used to code for the identity of different

10   primer pairs. An example for photocleavage and detection of four tags is shown in Figure 9 which shows APCI mass spectra for four mass tags after from the corresponding primers (mass tag # 1, 2-nitrosoacetophenone, m/z 150; mass tag # 2, 4-fluoro-2-nitrosoacetophenone, m/z 168;

15   mass tag # 3, 5-methoxy-2-nitrosoacetophenone, m/z 180; mass tag # 4, 4,5-dimethoxy-2-nitrosoacetopheone, m/z 210). The four mass tag-labeled primers were mixed together and the mixture was irradiated under UV light (λ~340 nm) for 5 seconds, introduced into an APCI mass

20   spectrometer and analyzed for the four masses to produce the above spectrum. The peak with m/z of 150 is mass-tag 1, 168 is mass-tag 2, 180 is mass-tag 3 and 210 is mass-tag 4. The mechanism for release of these tags from DNA is shown in Fig. 10 - Four mass tag-labeled DNA molecules

25   (Bottom) Chemical structures of the corresponding photocleaved mass tags (2-nitrosoacetophenone, 4-fluoro-2-nitrosoacetophenone, 5-methoxy-2-nitrosoacetophenone and 4,5-dimethoxy-2-nitrosoacetophenone) after UV irradiation at 340 nm. This result indicates that the 4

30   compounds designed as mass tags are stable and produce discrete high-resolution digital data in an APCI mass spectrometer. The unique m/z from each mass tag

translates to the identity of a viral sequence. In a recent collaboration with Qiagen, which has used a library of mass tags to discriminate up to 25 SNPs (Kokoris et al. 2000), we have significantly expanded the

5   number of the mass tags.

Establishment of a PCR/MS Assay for Respiratory Pathogens

During the SARS 2003 Beijing outbreak we established a

10   specific and sensitive real time PCR assay for SARS-CoV (Zhai et al, 2004). The assay was extended to allow simultaneous detection of SARS-CoV as well as human coronaviruses OC43 and 229E in light of recent data from China suggesting the potential for coinfection and

15   increased morbidity (Fig. 11). This human coronavirus assay (3 viral genes and 1 housekeeping gene) exhausted the repertoire of fluorescent tags with which to pursue multiplex real time PCR analysis of clinical materials. The importance of extending rapid molecular assays to

20   include other respiratory pathogens is reinforced by the reappearance of SARS in China and reports of a new highly virulent influenza virus strain in Vietnam.

To build a more comprehensive respiratory pathogen

25   surveillance assay we adapted the human coronavirus primers to the PCR/MS platform, and added reagents required to detect other relevant microbes. Influenza A virus was included through a set of established primer sequences obtained through Georg Pauli (Robert Koch

30   Institute, Germany; Schwaiger et al 2000). For the bacterial pathogen *M. pneumoniae* we also used unmodified primer sequences published for real time PCR (Welti et al

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2003) to evaluate their use on the PCR/MS platform. Using a panel of mass tags developed by QIAGEN, experiments were performed demonstrating the feasibility of detecting several respiratory pathogens in a single multiplexed

5   assay on the PCR/MS platform.

The current Masscode™ photocleavable mass tag repertoire comprises over 80 tags. Fig. 12 demonstrates the specificity of the mass tag detection approach in an

10   example where 58 different mass tags conjugated to oligonucleotides via a photocleavable linkage were identified after UV cleavage and MS. Each of the 10 primers for the 5-plex assay (SARS-CoV, CoV-229E, CoV-OC43, Influenza A virus, and *M. pneumoniae*) was

15   conjugated to a different mass tag such that the identity of a given pathogen was encoded by a specific binary signal (e.g. SARS-CoV, forward primer, 527 amu; reverse primer 666 amu; see Fig. 13B).

20   The presence of mass tags did not impair performance of primers in PCR and yielded clear signals for all 5 agents (Fig. 13A, 13B - Singleplex mass tag PCR for (1) Influenza A virus matrix protein (618 amu fwd-primer, 690 amu rev-primer), human coronaviruses (2) SARS (527/666),

25   (3) 229E (670/558), (4) OC43 (686/548), and the bacterial agent (5) M. pneumoniae (602/614). (6) 100 bp ladder). No noise was observed using unmodified or mass tag-modified primer sets in a background of 125 ng of normal total human DNA per assay (Fig. 13C). In subsequent experiments

30   we extended the respiratory pathogen panel to include respiratory syncytial virus groups A and B. Non-optimized pilot studies in this 7-plex system indicated a detection

threshold of <500 molecules. As a test of feasibility for PCR/MS detection of coinfection, mixtures of DNA templates representing two different pathogens were analyzed successful detection of two targets confirmed
5 the suitability of this technology for clinical applications where coinfection may be critical to pathogenesis and epidemiology.

Establishment of a platform for portable MS

10

Griffin has developed a portable mass spectrometer that is roughly the size of a tower computer (including vacuum system), weighs less than 50 lbs, and consumes ~150 W depending on operating conditions. This system has a mass
15 range of 400 Da with unit mass resolution. It has been used to detect part-per-trillion level atmospheric constituents. Figure 14 shows a representative spectrum of methyl salicylate collected on a miniature cylindrical ion trap mass analyzer coupled to a corona discharge
20 ionization source (data collected in Prof. R. G. Cooks research laboratory at Purdue University). This data demonstrates the feasibility of using this type of instrumentation to detect the mass tags of interest as well as the specificity of the ionization source. Fig. 14
25 shows mass spectrum representative of data collected using a miniature cylindrical ion trap mass analyzer coupled with a corona discharge ionization source.

Figure 15 shows a mass spectrum of perflouro-
30 dimethclcyclohexane collected on a prototype atmospheric sampling glow discharge ionization (ASGDI) source. ASGDI is an external ionization source related to the APCI

source discussed here.

Experimental Design

5   Labeled amplification products are generated during PCR amplification with mass tagged primers. After isolation from non-incorporated primers by binding to silica in Qiagen 96-well or 384-well PCR purification modules, products are eluted into the injection module of the

10   mass-spectrometer. The products traverse the path of a UV light source prior to entering the nebulizer, releasing photocleavable tags (one each from the forward and reverse primer). Mass tags are then ionized. Analysis of the mass code spectrum defines the pathogen composition

15   of the specimen.

A non-comprehensive list of target pathogens is listed in Tables 2 and 3. Forward and reverse primer pairs for pathogens listed in Table 2 are (reading from top to

20   bottom starting with RSV-A and ending with M. Pneumoniae), SEQ ID NOS:1 and 2, 3 and 4, 9 and 10, 21 and 22, 23 and 24, 26 and 27, and 49 and 50.

Table 2:	Respiratory Panel Mass-Tag Primers			
Pathogen	Forward primer	Sequence	Reverse primer	Sequence
RSV A	RSA-U1137	AgATCAACTTCTgTC ATCCAgCAA	RSV-L1192	gCACATCATAATTAggAg TATCAAT
RSV B	RSB-U1248	AAgATgCAAATCAT AAATTCACAggA	RSV-1318	TgATATCCAgCATCTTTA AgTATCTTTATAgTg
Influenza A (N1)				
Influenza A (N2)				
Influenza A (M)	AM-U151	CATggAATggCTAAA. gACAAgACC	AM-L397	AAgTgCACCAgCAgAATA ACTgAg
Influenza B			.	
SARS-CoV	CIID-28891F	AAg CCT CgC CAA AAA CgT AC	CIID-29100R	AAg TCA gCC ATg TTC CCg AA
229E-CoV	Taq-Co22-418F	ggC gCA AgA ATT CAg AAC CA	Taq-Co22-636R	TAA gAg CCg CAg CAA CTg C
OC43-CoV	Taq-Co43-270F	TgT gCC TAT TgC ACC Agg AgT	Taq-Co43-508R	CCC gAT CgA CAA TgT CAg C
Metapneumo virus				
Parainfluenza 1				
Parainfluenza 2				
Parainfluenza 3				
Parainfluenza 4				
M. pneumoniae	MTPM1	CCAACCAAACAACA ACgTTCA	MTPM2	ACCTTgACTggAggCCgTT A
L. pneumophi				

lae				
C. pneumoniae				

Design and Synthesis of Primers

5   Primers are designed using the same approach as employed for the 7-plex assay. Available sequences are be extracted from GenBank. Conserved regions suitable for primer design are identified using standard software programs as well as custom software (patent application XYZ). Primer

10   properties can be assessed by commercial primer selection software including OLIGO (Molecular Biology Insights), Primer Express (PE Applied Biosystems), and Primer Premiere (Premiere Biosoft International). Primers are evaluated for signal strength and specificity against a

15   background of total human DNA.

Isolation and Cloning of Template Standards

Targeted genes can be cloned into the transcription

20   vector pGEM-Teasy (Invitrogen) by conventional RT-PCR cloning methods. Quantitated plasmid standards are used in initial assay establishment. Thereafter, RNA transcripts generated by *in vitro* transcription, quantitated and diluted in a background of random human

25   RNA (representing brain, liver, spleen, lung and placenta in equal proportions) are employed to establish sensitivity and specificity parameters of RT-PCR/MS assays. One representative isolate for each targeted

pathogen/gene is used during initial establishment of the assay.

Inherent in the exquisite sensitivity of PCR is the risk

5   of false positive results due to inadvertent introduction of synthetic templates such as those comprising positive control and calibration reagents, and so calibration reagents are preferred components of kits. Thus, to allow recognition   of   control   vs   authentic,   natural

10 amplification products, calibration reagents are modified by introducing a restriction enzyme cleavage site in between the primer binding sites through site directed mutagenesis. This approach has been employed in projects concerned with epidemiology of viral infection in various

15 chronic   diseases   including   Bornaviruses   in neuropsychiatric disease (NIH/MH57467), measles virus in autism   (CDC/American   Academy   of   Pediatrics),   and enteroviruses in type I diabetes mellitus (NIH/AI55466).

20 *Multiplex Assay Using Cloned Template Standards*

Initially, the performancance of individual primer sets with unmodified primers is tested. Amplification products in   these   single   assays   canbe   detected   by   gel

25 electrophoresis.   This   strategy   will   not   serve   for multiplex assays because products of individual primer sets will be similar in size i.e. <300 bp. Thus, after confirmation of performance in single assays, mass tagged primers are generated for multiplex analyses. All assays

30 are first optimized for PCR using serial dilutions of plasmid DNA, and then for RT-PCR using serial dilutions of synthetic transcripts. A multiplex assay is considered

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successful if it detects all target sequences at a sensitivity of 50 copies plasmid DNA per assay and 100 copies RNA per assay. Successful multiplex assay performance includes detection of all permutative
5   combinations of two agents to ensure the feasibility of diagnosing simultaneous infection.

Optimizing Multiplex Assay Using Cell Culture Extracts

10   After establishing performance parameters with calibrated synthetic reagents, cell culture extracts of authentic pathogens are used. Performance of assays with RNA extracted using readily available commercial systems that do or do not include organic solvents (e.g, Tri-Reagent
15   vs RNeasy) is assessed. A protocol disclosed here employs Tri-Reagent. Similarly, although Superscript reverse transcriptase (Invitrogen) and HotStart polymerase (QIAGEN) can be used, performance of ThermoScript RT (Invitrogen) at elevated temperature can be assessed, as
20   are single-step RT-PCR systems like the Access Kit (Promega). To optimize efficiency where clinical material mass is limited and to reduce the complexity of sample preparation, both viral and bacterial agents can be identified using RT-PCR. Where an agent is characterized
25   by substantive phylogenetic diversity, cell culture systems should include at least three divergent isolates of each pathogen

Sample Processing

30

Samples may be obtained by nasal swabs, sputum and lavage specimens will be spiked with culture material to optimize recovery methods for viral as well as bacterial

agents.

Portable APCI MS instruments to support multiplex PCR/MS platform

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The multiplex mass tag approach is well-suited to implementation on a miniaturized MS system, as the photocleavable mass tags are all relatively low in molecular weight (<500 Da.), and hence the constraints on
10   the mass spectrometer in terms of mass range and mass resolution are not high. The technical challenge associated with this approach is the development of an atmospheric-pressure chemical ionization (APCI) source for use on a miniaturized MS to generate the mass tag
15   ions. Such a source has been coupled with a miniaturized MS in an academic setting.

Detection of NIAD Category A, B, and C Priority Agents

20   Using the same approach as outlined for respiratory pathogen detection, a multiplex assay for detection of selected NIAD Category A, B, and C priority agents can be created (Table 3). Primers and PCR conditions for several agents are already established and can be adapted to the
25   PCR/MS platform.

5

Table 3: NIAD Priority Agents
B. anthracis
Dengue viruses
West Nile virus
Japanese encephalitis virus
St. Louis encephalitis virus
Yellow Fever virus
La Crosse virus
California encephalitis virus
Rift Valley Fever virus
CCHF virus
VEE virus
EEE virus
WEE virus
Ebola virus
Marburg virus
LCMV
Junin virus
Machupo virus
Variola virus

Example 3

Background

5   Efficient laboratory diagnosis of infectious diseases is
       increasingly important to clinical management and public
       health. Methods for direct detection of nucleic acids of
       microbial pathogens in clinical specimens are rapid,
       sensitive and may succeed where fastidious requirements
10   for agent replication confound cultivation. Nucleic acid
       amplification systems are indispensable tools in HIV and
       HCV diagnosis, and are increasingly applied to pathogen
       typing, surveillance, and diagnosis of acute infectious
       disease. Clinical syndromes are only infrequently
15   specific for single pathogens; thus, assays for
       simultaneous consideration of multiple agents are needed.
       Current multiplex assays employ gel-based formats where
       products are distinguished by size, fluorescent reporter
       dyes that vary in color, or secondary enzyme
20   hybridization assays. Gel-based assays are reported that
       detect 2-8 different targets with sensitivities of 2-100
       pfu or <1-5 pfu, depending on whether amplification is
       carried out in a single or nested format, respectively
       (Ellis and Zambon 2002, Coiras et all. 2004).
25   Fluorescence reporter systems achieve quantitative·
       detection with sensitivity similar to nested
       amplification; however, their capacity to simultaneously
       query multiple targets is limited to the number of
       fluorescent emission peaks that can be unequivocally
30   separated. At present up to four fluorescent reporter
       dyes are detected simultaneously (Vet et al. 1999,
       Verweij et al. 2004). Multiplex detection of up to 9

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pathogens was achieved in hybridization enzyme systems; however, the method requires cumbersome post-amplification processing (Gröndahl et al. 1999).

5   To address the need for sensitive multiplex assays in diagnostic molecular microbiology we created a polymerase chain reaction (PCR) platform wherein microbial gene targets are coded by 64 distinct mass tags. Here we describe this system, mass tag PCR, and demonstrate its
10   utility in differential diagnosis of respiratory tract infections.

Oligonucleotide primers for mass tag PCR were designed to detect the broadest number of members for a given
15   pathogen species through efficient amplification of a 50-300 basepair product. In some instances we selected established primer sets; in others we employed a software program designed to cull sequence information from GenBank, perform multiple alignments, and maximize
20   multiplex performance by selecting primers with uniform melting temperatures and minimal cross-hybridization potential. Primers, synthesized with a 5' C6-spacer and aminohexyl modification, were covalently conjugated via a photocleavable linkage to small molecular weight tags
25   (Kokoris et al. 2000) to encode their respective microbial gene targets. Forward and reverse primers were labeled with differently sized tags to produce a dual code for each target that facilitates assessment of signal specificity.
30

Microbial gene target standards for sensitivity and specificity assessment were cloned by PCR using cDNA

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template obtained by reverse transcription of extracts from infected cultured cells or by assembly of overlapping synthetic polynucleotides. Cloned standards representing genetic sequence of the targeted microbial
5   pathogens were diluted in 12.5 ug/ml human placenta DNA (Sigma, St. Louis, MO, USA) and subjected to multiplex PCR amplification using the following cycling protocol: 9x C for X sec., 55 C for X sec., 72 C for X sec.; 50 cycles, MJ PTC200 (MJ Research, Waltham, MA, USA).
10   Amplification products were purified using QIAquick 96 PCR purification cartridges (Qiagen, Hilden, Germany) with modified binding and wash buffers (RECIPES). Mass tags of the amplified products were analyzed after ultraviolet photolysis and positive-mode atmospheric
15   pressure chemical ionization (APCI) by single quadrapole mass spectrometry. Figure 1 indicates discrimination of individual microbial targets in a 21-plex assay comprising sequences of 16 human pathogens. The threshold of detection met or exceeded 500 molecules corresponding
20   in sensitivity to less than 0.1 $TCID_{50}$/ml (0.001 $TCID_{50}$/assay), in titered cell culture virus of coronaviruses as well as parainfluenza viruses (data not shown). For 19 of 21 microbial targets the detection threshold was less than 100 molecules (Table 4).
25

We next analyzed samples from individuals with respiratory infection using a larger panel comprising 30 gene targets (26 pathogens). Mass Tag PCR correctly identified infection with respiratory syncitial, human
30   parainfluenza, SARS corona, adeno, entero, metapneumo and influenza viruses (Table 4 and Figure 16). A smaller panel comprising 18 gene targets (18 central nervous

system pathogens) was used to analyze cerebrospinal fluid from individuals with meningitis or encephalitis. Two of four cases of West Nile virus encephalitis were identified. Fifteen of seventeen cases of enteroviral
5   meningitis were detected representing serotypes CV-B2, CV-B3, CV-B5, E-6, E-11, E-13, E-18, and E-30.

Our results indicate that mass tag PCR is a useful method for molecular characterization of microflora. Sensitivity
10 is similar to real time PCR assays but with the advantage of allowing simultaneous screening for several candidate pathogens. Potential applications include differential diagnosis of infectious diseases, blood product surveillance, forensic microbiology, and biodefense.
15

Figure 16 shows the sensitivity of 21-plex mass tag PCR. Dilutions of cloned gene target standards (10 000, 1 000, 500, 100 molecules/assay) diluted in human placenta DNA were analyzed by mass tag PCR. Each reaction mix
20 contained 2x Multiplex PCR Master Mix (Qiagen), the indicated standard and 42 primers at 1X nM concentration labeled with different mass tags. Background in reactions without standard (no template control, 12.5 ng human DNA) was subtracted and the sum of Integrated Ion Current for
25 both tags was plotted.

Figure 17 shows analysis of clinical specimens. (A) Respiratory infection; (B) Encephalitis. RNA from clinical specimens was extracted by standard procedures
30 and reverse transcribed into cDNA (Superscript RT system, Invitrogen, Carlsbad, CA; 20 ul volume). Five microliter of reaction was then subjected to mass tag PCR. (A)

Detection of Influenza A (H1N1), RSV-B, SARS-CoV, HPIV-3, HPIV-4, and ENTERO using a 31-plex assay including 64 primers targeting Influenza A virus (FLUAV) matrix gene, and for typing H1, H2, H3, H5, N1, and N2 sequence, as well as influenza B virus (FLUBV), respiratory syncytial virus (RSV) groups A and B, human coronaviruses 229E, OC43, and SARS (HCoV-229E, -OC43, and -SARS), human parainfluenza virus (HPIV) types 1, 2, 3, and 4 (groups A and B combined), metapneumovirus, enteroviruses (EV, targeting all serogroups), adenoviruses (HAdV, targeting all serogroups), Mycoplasma pneumoniae, Chlamydia pneumoniae, Legionalla pneumophila, Streptococcus pneumoniae, Haemophilus influenzae, Human herpesvirus 1 (HHV-1, Herpes simplex virus), Human herpesvirus 3 (HHV-3; Varicella-zoster virus), Human herpesvirus 5 (HHV-5, Human cytomegalovirus), Human immunodeficiency virus 1 (HIV-1) and Human immunodeficiency virus 1HIV-2. (B) Detection of ENTERO XX, YY, and ZZ using an 18-plex assay including 36 primers targeting FLUAV matrix gene, H1, H2, H3, H5, N1, and N2 sequence, FLUBV, HCoV 229E, OC43, and SARS, EV, HAdV, HHV-1, -3, and -5, HIV-1, and -2, measles virus (MEV), West Nile virus (WNV), St. Louis virus (SLEV), S. pneumoniae, H. influenzae, and Neisseria meningitides.

Influenza A Matrix	Influenza A N1	Influenza A N2	Influenza A HA1	Influenza A HA2	Influenza A HA3	Influenza A HA5	Influenza B HA	RSV Group A	RSV group B	Metapneumo virus
100	100	100	100	100	100	100	500	100	100	100
CoV-SARS	CoV-OC43	CoV-229E	HPIV-1	HPIV-2	HPIV-3	C. pneumoniae	M. pneumoniae	L. pneumophila	Enterovirus (genus)	Adenovirus (genus)
100	100	100	100	100	100	100	100	100	5 000	5 000

Table 4. Sensitivity of 22-plex mass tag PCR. Numbers in cells indicate target copy threshold.

Example 4

Multiplex PCR

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Conventional multiplex PCR assays are established, however, none allow sensitive detection of more than 10 genetic targets. The most sensitive of these assays, real time PCR, is limited to four fluorescent reporter dyes.

10   Gel based systems are cumbersome and limited to visual distinction of products that differ by 20 bp; multiplexing is restricted to the number of products that can be distinguished at 20 bp intervals within the range of 100 to 250 bp (amplification efficiency decreases with

15   larger products); nesting or Southern hybridization is required for high sensitivity. A 9-plex assay has been achieved using hybridization capture enzyme assay.

Disclosed here are panels of nucleic acid sequences to be

20   used in assays for the detection of infectious agents. The sequences include primers for polymerase chain reaction, enzyme sites for initiating isothermal amplification, hybridization selection of nucleic acid targets, as well as templates to serve as controls for

25   validation of these assays. This example focuses on the use of these panels for multiplex mass tag PCR applications. Nucleic acid databases were queried to identify regions of sequence conservation within viral and bacterial taxa wherein primers could be designed that

30   met the following critera: (i) the presence of motifs required to create specific or low degeneracy PCR primers that targeted all members of a microbial group (or

subgroup); (ii) Tm of 59-61 C; (iii) GC content of 48-
60%; (iv) length of 18-24 bp; (v) no more than three
consecutive identical bases; (vi) 3 or more G and/or C
residues in the 5'-hexamer; (vii) less than 3 G and/or C
5   residues in the 3'-pentamer; (vii) no propensity for
secondary structure (stem-loop) formation; (viii) no
inter-primer complementarity that could predispose to
primer-dimer formation; (ix) amplification of an 80- 250
bp region with no or little secondary structure at 59-61
10   C. Primers meeting these criteria were then evaluated
empirically for equal performance in context of the
respective multiplex panel. In the event that no ideal
primer candidates could be identified, primers that did
not meet one or more of these criteria were synthesized
15   and evaluated for appropriate performance. Those that
yielded 80-250 bp amplification products, had Tm of 59-61
C, and showed no primer-dimer artifacts were selected for
inclusion into panels.

20   As a proof-of-principle we designed a panel of primers
for detection of 31 target sequences of respiratory
pathogens (25-plex respiratory panel) and demonstrated
successful detection of all potential targets in a 25-
plex PCR reaction. Detection of amplification products
25   was achieved through use of the MASSCODE® technology.
Individual primers were conjugated with a unique masscode
tag through a photocleavable linkage. Photocleavage of
the masscode tag from the purified PCR product and mass
spectrometric analysis identifies the amplified target
30   through the two molecular weights assigned to the forward
and reverse primer. Primer panels focus on groups of

infectious pathogens that are related to differential diagnosis of respiratory disease, encephalitis, or hemorrhagic fevers; screening of blood products; biodefense; food safety; environmental contamination; or
5   forensics.

Example 5

Background and Significance

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The advent of SARS in 2003 poignantly demonstrated the urgency of establishing rapid, sensitive, specific, inexpensive tools for differential laboratory diagnosis of infectious diseases. Through unprecedented global

10   collaborative efforts, the causative agent was rapidly implicated and characterized, facilitating development of serologic and molecular assays for infection, and containment of the outbreak. Nonetheless, as the northern hemisphere entered the winter season of 2004, the

15   diagnosis of SARS still rests on clinical and epidemiological as well as laboratory criteria. The WHO SARS International Reference and Verification Laboratory Network met on October 22, 2003 to review the status of laboratory diagnostics in acute severe pulmonary disease.

20   Quality assurance testing indicated that false positive SARS CoV PCR results were infrequent in network labs. However, participants registered concern that current assays did not allow simultaneous detection of a wide range of pathogens that could aggravate disease or

25   themselves result in clinical presentations similar to SARS.

Methods for cloning nucleic acids of microbial pathogens directly from clinical specimens offer new opportunities

30   to investigate microbial associations in diseases. The power of these methods is not only sensitivity and speed

but also the potential to succeed where methods for pathogen identification through serology or cultivation may fail due to absence of specific reagents or fastidious requirements for agent replication.

Various methods are employed or proposed for cultivation-independent characterization of infectious agents. These can be broadly segregated into methods based on direct analysis of microbial nucleic acid sequences, direct analysis of microbial protein sequences, immunological systems for microbe detection, and host response profiling. Any comprehensive armamentarium should include most, if not all, of these tools. Nonetheless, classical methods for microbiology remain important. Indeed, the critical breakthrough during the SARS outbreak was the cultivation of the agent in tissue culture.

Real-time PCR methods have significantly changed diagnostic molecular microbiology by providing rapid, sensitive, specific tools for detecting and quantitating genetic targets. Because closed systems are employed, real-time PCR is less likely than nested PCR to be confounded by assay contamination due to inadvertent aerosol introduction of amplicon/positive control/cDNA templates that can accumulate in diagnostic laboratories. The specificity of real time PCR is both, a strength and a limitation. Although the potential for false positive signal is low so is the utility of the method for screening to detect related but not identical genetic targets. Specificity in real-time PCR is provided by two primers (each approximately 20 matching nucleotides (nt)

in length) combined with a specific reporter probe of about 27 nt. The constraints of achieving hybridization at all three sites may confound detection of diverse, rapidly evolving microbial genomes such as those of

5   single-stranded RNA viruses. These constraints can be compensated in part by increasing numbers of primer sets accommodating various templates. However, because real-time PCR relies on fluorescent reporter dyes, the capacity for multiplexing is limited to the number of

10   emission peaks that can be unequivocally separated. At present up to four dyes can be identified simultaneously. Although the repertoire may increase, it will unlikely to change dramatically.

15   To address the need for enhanced multiplex capacity in diagnostic molecular microbiology we have established a PCR platform based on mass tag reporters that are easily distinguished in MS as discrete signal peaks. Major advantages of the PCR/MS system include: (1)

20   hybridization to only two sites is required (forward and reverse primer binding sites) vs real time PCR where an intermediate third oligonucleotide is used (probe binding site); this enhances flexibility in primer design; (2) tried and proven consensus PCR primers can be adapted to

25   PCR/MS; this reduces the time and resources that must be invested to create new reagents and assay controls; (3) the current repertoire of 60 tags allows highly multiplexed assays; additional tags can be easily synthesized to allow further complexity; and (4)

30   sensitivity of real time PCR is maintained. A limitation of PCR/MS is that it is unlikely to provide more than a

75

semi-quantitative index of microbe burden. Thus, we view PCR/MS as a tool with which to rapidly screen clinical materials for the presence of candidate pathogens. Thereafter, targeted secondary tests, including real time

5   PCR, should be used to quantitate microbe burden and pursue epidemiologic studies.

Selection of APCI LCMS Platform

10 Mass spectrometry is a rapid, sensitive method for detection of small molecules. With the development of Ionization techniques such as matrix assisted laser desorption ionization (MALDI) and electrospray ionization (ESI), MS has become a indispensable tool in many areas

15 of biomedical research. Although these ionization methods are suitable for the analysis of bioorganic molecules, such as peptides and proteins, improvements in both detection and sample preparation will be required before mass spectrometry can be used to directly detect long DNA

20 fragments. A major confound in exploiting MS for genetic investigation has been that long DNA molecules are fragmented during the analytic process. The mass tag approach we have developed overcomes this limitation by detecting small stable mass tags that serve as signatures

25 for specific DNA sequences rather than the DNA sequences themselves.

Ionization and detection of the photocleaved mass tags have been extensively characterized using atmospheric

30 pressure chemical ionization (APCI) as the ionization source while using a single quadrupole mass spectrometer as the detector (Jingyue et al. , Kim et al. 2003;

Kokoris et al. 2000). Because buffer and inorganic salts impact ionization efficiency, performance in ESI was determined to be critically dependent upon sample preparation conditions. In MALDI, matrix must be added
5   prior to sample introduction into the mass spectrometer, which is a time consuming step that requires costly sample spotting instrumentation. Similarly, speed is often limited by the need to search for an ideal irradiation spot to obtain interpretable mass spectra. In
10 contrast, APCI is much more tolerant of residual inorganic salts (than ESI) and does not require mixing with matrix to prepare crystals on a target plate. Thus, mass tag solutions can be injected directly into the MS via a Liquid Chromatography (LC) delivery system. Since
15 mass tags ionize well under APCI conditions and have small mass values (less that 800 amu), they are detected with high sensitivity (< 5 femtomolar limit of detection) with the APCI-Quadrupole LCMS platform.

20 Methods for synthesis and APCI-MS analysis of mass tags coupled to DNA fragments are illustrated in Figure 1 where precursors are (a) acetophenone; (b) 4-fluoroacetophenone; (c) 3-methoxyacetophenone; and (d) 3,4-dimethoxyacetophenone.
25

Upon nitration and reduction, the photoactive tags are produced and used to code for the identity of different primer pairs. An example for photocleavage and detection of four tags is shown in Figure 9. APCI mass spectra for
30 four mass tags after from the corresponding primers (*mass tag # 1*, 2-nitrosoacetophenone, m/z 150; *mass tag # 2*, 4-

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fluoro-2-nitrosoacetophenone, m/z 168; *mass tag # 3*, 5-methoxy-2-nitrosoacetophenone, m/z 180; *mass tag # 4*, 4,5-dimethoxy-2-nitrosoacetopheone, m/z 210). The four mass tag-labeled primers were mixed together and the

5   mixture was irradiated under UV light ($\lambda \sim 340$ nm) for 5 seconds, introduced into an APCI mass spectrometer and analyzed for the four masses to produce the spectrum. The peak with m/z of 150 is mass-tag 1, 168 is mass-tag 2, 180 is mass-tag 3 and 210 is mass-tag 4.

10

The mechanism for release of these tags from DNA is shown in Fig. 10. Four mass tag-labeled DNA molecules (Bottom) Chemical structures of the corresponding photocleaved mass tags (2-nitrosoacetophenone, 4-fluoro-2-

15   nitrosoacetophenone, 5-methoxy-2-nitrosoacetophenone and 4,5-dimethoxy-2-nitrosoacetophenone) after UV irradiation at 340 nm.

This result indicates that the 4 compounds designed as

20   mass tags are stable and produce discrete high-resolution digital data in an APCI mass spectrometer. In the research plan described below, the unique m/z from each mass tag will translate to the identity of a viral sequence. Qiagen has developed a large library of more

25   than 80 proprietary masscode tags (Kokoris et al. 2000). Examples are shown in Figure 19.

Establishment of a PCR/MS assay for respiratory pathogens
During the SARS 2003 Beijing outbreak we established a

30   specific and sensitive real time PCR assay for SARS-CoV (Zhai et al, 2004). The assay was extended to allow

simultaneous detection of SARS-CoV as well as human coronaviruses OC43 and 229E in light of recent data from China suggesting the potential for coinfection and increased morbidity (Figure 11). This human coronavirus
5   assay (3 viral genes and 1 housekeeping gene) exhausted the repertoire of fluorescent tags with which to pursue multiplex real time PCR analysis of clinical materials. The importance of extending rapid molecular assays to include other respiratory pathogens is reinforced by the
10   reappearance of SARS in China and reports of a new highly virulent influenza virus strain in Vietnam.

To build a more comprehensive respiratory pathogen surveillance assay we adapted the human coronavirus
15   primers to the PCR/MS platform, and added reagents required to detect other relevant microbes. Influenza A virus was included through a set of established primer sequences obtained through Georg Pauli (Robert Koch Institute, Germany; Schwaiger et al 2000). For the
20   bacterial pathogen *M. pneumoniae* we also used unmodified primer sequences published for real time PCR (Welti et al 2003) to evaluate their use on the PCR/MS platform. Using a panel of mass tags developed by QIAGEN, pilot experiments were performed, demonstrating the feasibility
25   of detecting several respiratory pathogens in a single multiplexed assay on the PCR/MS platform.

Subsequent to the 1999 West Nile Virus (WNV) outbreak in the U.S. we also built a real time PCR assay for
30   differential diagnosis of flaviviruses WNV and St. Louis encephalitis virus - see Figure 20. Other validated tools

for broad range detection of NIAID priority agents
include universal primer stes for detection of Dengue
type 1, 2, 3, and 4; various primer sets detecting all
members of the bunyamwera and California encephalitis
5   serogroups of the bunyaviruses, see table 13, and not yet
validated primer sets for detection of all six Venezuelan
equine encephailitis virus serotypoes developed for
Molecular Epidemiology, AFEIRA/SDE. Brooks, TX.

10   The current Masscode photocleavable mass tag repertoire
comprises over 80 tags. Figure 12 demonstrates the
specificity of the mass tag detection approach in an
example where 58 different mass tags conjugated to
oligonucleotides via a photocleavable linkage were
15   identified after UV cleavage and MS. Each of the 10
primers for the 5-plex assay (SARS-CoV, CoV-229E, CoV-
OC43, Influenza A virus, and *M. pneumoniae*) was
conjugated to a different mass tag such that the identity
of a given pathogen was encoded by a specific binary
20   signal (e.g. SARS-CoV, forward primer, 527 amu; reverse
primer 666 amu; see Figure 13B). The presence of mass
tags did not impair performance of primers in PCR and
yielded clear signals for all 5 agents (Figures 13A,
13B). No noise was observed using unmodified or mass tag-
25   modified primer sets in a background of 125 ng of normal
total human DNA per assay (Figure 13C). In general,
Figure 13 shows singleplex mass tag PCR for (1) Influenza
A virus matrix protein (618 amu fwd-primer, 690 amu rev-
primer), human coronaviruses (2) SARS (527/666), (3) 229E
30   (670/558), (4) OC43 (686/548), and the bacterial agent
(5) M. pneumoniae (602/614). (6) 100 bp ladder. In

subsequent experiments we extended the respiratory pathogen panel to include respiratory syncytial virus groups A and B. Non-optimized pilot studies in this 7-plex system indicated a detection threshold of <500
5   molecules (Figure 21). As a test of feasibility for PCR/MS detection of coinfection, mixtures of DNA templates representing two different pathogens were analyzed successful detection of two targets (Figure 21) confirmed the suitability of this technology for clinical
10   applications where coinfection may be critical to pathogenesis and epidemiology.

Establishment of a platform for portable MS

15   Griffin has developed a portable mass spectrometer that is roughly the size of a tower computer (including vacuum system), weighs less than 50 lbs, and consumes ~150 W depending on operating conditions. This system has a mass range of 400 Da with unit mass resolution. It has been
20   used to detect part-per-trillion level atmospheric constituents. Included below is a representative spectrum of methyl salicylate collected on a miniature cylindrical ion trap mass analyzer coupled to a corona discharge ionization source (data collected in Prof. R. G. Cooks
25   research laboratory at Purdue University). This data demonstrates the feasibility of using this type of instrumentation to detect the mass tags of interest as well as the specificity of the ionization source. Figure 14 shows mass spectrum data representative of data
30   collected using a miniature cylindrical ion trap mass analyzer coupled with a corona discharge ionization

source. Figure 15 shows a mass spectrum of perflouro-dimethclcyclohexane collected on a prototype atmospheric sampling glow discharge ionization (ASGDI) source. ASGDI is an external ionization source related to the APCI

5   source proposed here.

Griffin has developed a mass spectrometer for field transportable use. Power consumption, weight, size, and

10   ease of use have been focus design points in the development of this instrument. It has not been designed specifically for interface to an atmospheric pressure ionization (API) source like the one proposed here for pathogen surveillance and discovery. Thus, our focus in

15   this proposal is directed toward the integration of an atmospheric pressure chemical ionization (APCI) source and the required vacuum, engineering, and software considerations associated with this integration.

20   *Experimental Design*

A cartoon of the assay procedure is shown in Figure 22. Labeled amplification products will be generated during PCR amplification with mass tagged primers. After

25   isolation from non-incorporated primers by binding to silica in Qiagen 96-well or 384-well PCR purification modules, products will be eluted into the injection module of the mass-spectrometer. The products traverse the path of a UV light source prior to entering the

30   nebulizer, releasing photocleavable tags (one each from the forward and reverse primer). Mass tags are then ionized. Analysis of the mass code spectrum defines the

pathogen composition of the specimen.

The repertoire of potential pathogens to be targeted
during this project is listed in Table 13. Forward and
5   reverse primer pairs for pathogens listed in Table 13 are
(reading from top to bottom starting with RSV-A and
ending with M. Pneumoniae), SEQ ID NOS:1 and 2, 3 and 4,
9 and 10, 21 and 22, 23 and 24, 26 and 27, and 49 and 50.

Table 13:	Respiratory Panel Mass-Tag Primers			
Pathogen	Forward primer	Sequence	Reverse primer	Sequence
RSV A	RSA-U1137	AgATCAACTTCTgTCATCCAgCAA	RSV-L1192	gCACATCATAATTAggAgTATCAAT
RSV B	RSB-U1248	AAgATgCAAATCATAAATTCACAggA	RSV-1318	TgATATCCAgCATCTTTAAgTATCTTTATAgTg
Influenza A (N1)				
Influenza A (N2)				
Influenza A (M)	AM-U151	CATggAATggCTAAAgACAAgACC	AM-L397	AAgTgCACCAgCAgAATAACTgAg
Influenza B				
SARS-CoV	CIID-28891F	AAg CCT CgC CAA AAA CgT AC	CIID-29100R	AAg TCA gCC ATg TTC CCg AA
229E-CoV	Taq-Co22-418F	ggC gCA AgA ATT CAg AAC CA	Taq-Co22-636R	TAA gAg CCg CAg CAA CTg C
OC43-CoV	Taq-Co43-270F	TgT gCC TAT TgC ACC Agg AgT	Taq-Co43-508R	CCC gAT CgA CAA TgT CAg C
Metapneumovirus				
Parainfluenza 1				
Parainfluenza 2				
Parainfluenza 3				
Parainfluenza 4				
M. pneumoniae	MTPM1	CCAACCAAACAACAACgTTCA	MTPM2	ACCTTgACTggAggCCgTTA
L. pneumophilae				
C. pneumoniae				

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83

Design and synthesize primers

Missing primers will be designed using the same approach
as employed for the 7-plex assay. Available sequences
will be extracted from GenBank. Conserved regions
suitable for primer design will be identified using
standard software programs as well as custom software
(patent application XYZ). Primer properties will be
assessed by commercial primer selection software
including OLIGO (Molecular Biology Insights), Primer
Express (PE Applied Biosystems), and Primer Premiere
(Premiere Biosoft International). Non-tagged primers will
be synthesized, and performance assessed using cloned
target sequences as described in preliminary data.
Primers will be evaluated for signal strength and
specificity against a background of total human DNA.
Currently, 80% of primers perform as predicted by our
algorithms. Thus, to minimize delay we typically
synthesize multiple primer sets for similar genetic
targets and evaluate their performance in parallel.

Inherent in the exquisite sensitivity of PCR is the risk
of false positive results due to inadvertent introduction
of synthetic templates such as those comprising positive
control and calibration reagents. Calibration reagents
will be components of kits distributed to network
laboratories and customers. Thus, to allow recognition of
control vs authentic, natural amplification products, we
will modify calibration reagents by introducing a
restriction enzyme cleavage site in between the primer
binding sites through site directed mutagenesis. We have

used this approach in projects concerned with epidemiology of viral infection in various chronic diseases including Bornaviruses in neuropsychiatric disease (NIH/MH57467), measles virus in autism

5   (CDC/American Academy of Pediatrics), and enteroviruses in type I diabetes mellitus (NIH/AI55466).

Establish multiplex assay using cloned template standards

10   Before committing resources to generating mass tagged primers we will test the performance of individual primer sets with unmodified primers. Amplification products in these single assays will be detected by gel electrophoresis. This strategy will not serve for

15   multiplex assays because products of individual primer sets will be similar in size i.e., all will be <300 bp. Although individual products in multiplex assays could be resolved by sequence analysis our experience suggests it will be more cost effective to proceed directly to PCR/MS

20   analysis. Thus, after performance is confirmed in single assays we will generate mass tagged primers for multiplex analyses. All assays will be optimized first for PCR using serial dilutions of plasmid DNA, and then for RT-PCR using serial dilutions of synthetic transcripts. A

25   multiplex assay will be considered successful if it detects all target sequences at a sensitivity of 50 copies plasmid DNA per assay and 100 copies RNA per assay. Successful multiplex assay performance will also include detection of all permutative combinations of two

30   agents to ensure the feasibility of diagnosing simultaneous infection.

Optimize multiplex assay using cell culture extracts

After establishing performance parameters with calibrated
synthetic reagents, cell culture extracts of authentic
pathogens will be used. We will recommend specific kits
for nucleic acid extraction and RT-PCR. Nonetheless, we
recognize that some investigators may choose to use other
reagents. Thus, we will assess performance of assays with
RNA extracted using readily available commercial systems
that do or do not include organic solvents (e.g, Tri-
Reagent vs RNeasy). Our current protocol employs Tri-
Reagent. Similarly, although we use Superscript reverse
transcriptase (Invitrogen) and HotStart polymerase
(QIAGEN), we will also assess the performance of
ThermoScript RT (Invitrogen) at elevated temperature, and
of single-step RT-PCR systems like the Access Kit
(Promega). To optimize efficiency where clinical material
mass is limited and to reduce the complexity of sample
preparation, both viral and bacterial agents will be
identified using RT-PCR. In the event network
collaborators agree an agent is characterized by
substantive phylogenetic diversity, cell culture systems
will include at least three divergent isolates of each
pathogen. Nasal swabs, sputum and lavage specimens will
be spiked with culture material to optimize recovery
methods for viral as well as bacterial agents. Assays are
validated using banked specimens from naturally infected
humans, and naturally infected animals.

References for Example 5

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10

Example 6

5   *Primer design and synthesis, template design and synthesis*

Respiratory Panel includes 27 gene targets with validated primer sets as shown below in Table 5.
10   Forward and reverse primer pairs (SEQ ID NOs:1-54) are given for each pathogen (reading from top to bottom starting with RSV-A and ending with C. Pneumoniae). For example, forward primer for RSV-A is SEQ ID NO:1, reverse primer for RSV-A is SEQ ID NO:2. Forward primer
15   for RSV-B is SEQ ID NO:3, reverse primer for RSV-B is SEQ ID NO:4, etcetera.

Table 5:	Respiratory Panel Mass-Tag Primers				
Pathogen	Forward primer	Sequence	Reverse primer	Sequence
RSV A	RSA-U1137	AgATCAACTTCTgTCATCCAgCAA	RSV-L1192	gCACATCATAATTAggAgTATCAAT
RSV B	RSB-U1248	AAgATgCAAATCATAAATTCACAggA	RSV-1318	TgATATCCAgCATCTTTAAgTATCTTTATAgTg
Influenza A (N1)	NA1-U1078	ATggTAATggTgTTTggATAggAAg	NA1-L1352	AATgCTgCTCCCACTAgTCCAg
Influenza A (N2)	NA2-U560	AAgCATggCTgCATgTTTTgTg	NA2-L858	ACCAggATATCgAggATAACAggA
Influenza A (M)	AM-U151	CATggAATggCTAAAgACAAgACC	AM-L397	AAgTgCACCAgCAgAATAACTgAg
Influenza A (H1)	HA1-U583	ggTgTTCATCACCCgTCTAACAT	HA1-L895	gTgTTTgACACTTCgCgTCACAT
Influenza A (H2)	H2A208U27	gCTATgCAAACTAAACggAATYCCTCC	H2A559L26	TATTgTTgTACgATCCTTTggCAACC
Influenza A (H3)	HA3-U115	gCTACTgAgCTggTTCAgAgTTC	HA3-L375	gAAgTCTTCATTgATAAACTCCAg
Influenza A (H5)	HA5human-u71	TTACTgTTACACATgCCCAAgACA	HA5human-L147	AggYTTCACTCCATTTAgATCgCA
Influenza B	BHA-U188	AgACCAgAgggAAACTATgCCC	BHA-L347	CTgTCgTgCATTATAggAAAgCAC
SARS-CoV	CIID-28891F	AAgCCTCgCCAAAAACgTAC	CIID-29100R	AAgTCAgCCATgTTCCCgAA
229E-CoV	Taq-Co22-418F	ggCgCAAgAATTCAgAACCA	Taq-Co22-636R	TAAgAgCCgCAgCAACTgC
OC43-CoV	Taq-Co43-270F	TgTgCCTATTgCACCAggAgT	Taq-Co43-508R	CCCgATCgACAATgTCAgC
Metapneumovirus European	MPV01.2	AACCgTgTACTAAgTgATgCACTC	MPV02.2	CATTgTTTgACCggCCCCATAA
Metapneumovirus Canadian	MV-Can-U918	AAgTCCAAAggCAggRCTgTTATC	MV-Can-L992	CCTgAAgCATTRCCAAgAACAACAC
Parainfluenza 1	HPIV1-U82	TACTTTTgACACATTTAgTTCCAggAg	HPIV1-L167	CggTACTTCTTTgACCAggTATAATTg
Parainfluenza 2	HPIV2-U908	ggACTTggAACAAgATggCCT	HPIV2-L984	AgCATgAgAgCYTTTAATTTCTggA
Parainfluenza 3	HPIV3-U590	gCTTTCAgACAAgATggAACAgTg	HPIV3-L668	gCATKATTgACCCAATCTgATCC
Parainfluenza 4A	HPIV4A-U191	AACAgAAggAAATgATggTggAAC	HPIV4A-L269	TgCTgTggATgTATgggCAg
Parainfluenza 4B	HPIV4B-U194	AgAAgAAAACAACgATgAgACAAgg	HPIV4B-L306	gTTTCCCTggTTCACTCTCTTCA
Cytomegalovirus	CMV-U421	TACAgCACgCTCAACACCAACgCCT	CMV-L501	CCCggCCTTCACCACCAACCgAAAA
Measles virus	MEA-U1103	CAAgCATCATgATYgCCATTCCTgg	MEA-L1183	CCTgAATCYCTgCCTATgATgggTTT
Adenovirus	ADV2F-A	CCCMTTYAACCACCACCg	ADV1R-A	ACATCCTTBCKgAAgTTCCA
Enterovirus	5UTR-U447	TCCTCCggCCCCTgAATgCggCTAATCC	5UTR-L541	gAAACACggWCACCCAAAgTASTCg
M. pneumoniae	MTPM1	CCAACCAAACAACAACgTTCA	MTPM2	ACCTTgACTggAggCCgTTA
L. pneumophilae	Legpneu-U149	gCATWgATgTTARTCCggAAgCA	LegPneu-L223	CggTTAAAgCCAATTgAgCg
C. pneumoniae	CLPM1	CATggTgTCATTCgCCAAgT	CLPM2	CgTgTCgTCCAgCCATTTTA

Table 6, NIAID Priority Agent Panel.

Assays have been designed using 4 primer sets and their cognate synthetic Rift Valley Fever, Crimean Congo Hemorrhagic Fever, Ebola Zaire and Marburg virus

templates created via PCR using overlapping polynucleotides, as shown in Table 6. Forward and reverse primer pairs (SEQ ID NOs:55-62) are given for four of the listed pathogens (reading from top to

5   bottom starting with Rift Valley Fever virus and ending with Marburg virus). For example, forward primer for Rift Valley Fever virus is SEQ ID NO:55, reverse primer for Rift Valley Fever virus is SEQ ID NO:56. Forward primer for CCHF virus is SEQ ID NO:57, reverse primer

10   for CCHF virus is SEQ ID NO:58, etcetera.

Table 6: NIAID Priority Agents Panel Mass-Tag Primers				
Pathogen	Forward primer	Sequence	Reverse primer	Sequence
B. anthracis				
Dengue viruses				
West Nile virus				
Japanese enc. virus				
St. Louis enc. virus				
Yellow Fever virus				
La Crosse virus				
California enc. virus				
Rift Valley Fever virus	RVF-L660	ggATTgACCTgTgCCTgTTgC	RVF-L660	gCATTAgAAATgTCCTCTTTTgCTgC
CCHF virus	CCHV-L120	AgAACACgTgCCgCTTACgCCCA	CCHV-L120	CCATTCYTTYTTRAACTCYTCAAACCA
VEE virus				
EEE virus				
WEE virus				
Ebola virus	EboZA-L319	AACACCgggTCTTAATTCTTATATCAA	EboZA-L319	ggTggTAAAATTCCCATAgTAgTTCTTT
Marburg virus	Mar-L372	TTCCgTCACAAgCCgAAATT	Mar-L372	TTATTTTAgTTgAgAAAAgAggTTCATgC
LCMV				
Junin virus				
Machupo virus				
Variola virus				

Encephalitis Agent Panel

15   Table 7 shows primer sets for encephalitis-inducing agents. Forward and reverse primer pairs (SEQ ID NOs:63-96) are given for each pathogen (reading from

top to bottom starting with West Nile virus and ending with Enterovirus). For example, forward primer for West Nile virus is SEQ ID NO:63, reverse primer for West Nile virus is SEQ ID NO:64. Forward primer for St.

5 Louis Encephalitis virus is SEQ ID NO:65, reverse primer for St. Louis Encephalitis virus is SEQ ID NO:66, etcetera.

Table 7:	Encephalitis Agent Panel Mass-Tag Primers			
Pathogen	Forward primer	Sequence	Reverse primer	Sequence
West Nile virus	DF3 -87F	gCTCCgCTgTCCCTgTgA	DF3 -156R	CACTCTCCTCCTgCATggATg
St. Louis enc. virus	SLE-D-73F	CATTTgTTCAgCTgTCCCAgTC	SLE-D-145R	CTCACCCTTCCCATgAATTgAC
Herpes Simplex virus	HSV-U27	CCCggATgCggTCCAgACgATTAT	HSV-L121	CCCgCggAggTTgTACAAAAAgCT
HIV 1	SK68i	TTCTTIggAgCAgCIggAAgCACIATgg	SK69i	TTMATgCCCCAgACIgTIAgTTICAACA
HIV 2	HIV2TMF PR2	ggCTgCACgCCCTATgATA	HIV2TMR PR2	TCTgCATggCTgCTTgATg
N. meningitidis	Nmen-U829	TCTgAAgCCATTggCCgT	Nmen-L892	CAAACACACCACgCgCAT
S. pneumoniae	SPPLY-U532	AgCgATAgCTTTCTCCAAgTgg	SPPLY-L606	CTTAgCCAACAAATCgTTTACCg
H. influenzae	HINF-U82	AAgCTCCTTgMATTTTTTgTATTAgAA	Hinf-L158	gCTgAATTggCTTRgATACCgAg
Influenza B	BHA-U188	AgACCAgAgggAAACTATgCCC	BHA-L347	CTgTCgTgCATTATAggAAAgCAC
SARS-CoV	CIID-28891F	AAgCCTCgCCAAAAACgTAC	CIID-29100R	AAgTCAgCCATgTTCCCgAA
229E-CoV	Taq-Co22-418F	ggCgCAAgAATTCAgAACCA	Taq-Co22-636R	TAAgAgCCgCAgCAACTgC
OC43-CoV	Taq-Co43-270F	TgTgCCTATTgCACCAggAgT	Taq-Co43-508R	CCCgATCgACAATgTCAgC
Cytomegalovirus	CMV-U421	TACAgCACgCTCAACACCAACgCCT	CMV-L501	CCCggCCTTCACCACCAACCgAAAA
Varicella Zoster virus	VZV-U138	ACgTggATCgTCggATCAgTTgT	VZV-L196	TCgCTATgTgCTAAAACACgCgg
Measles virus	MEA-U1103	CAAgCATCATgATYgCCATTCCTgg	MEA-L1183	CCTgAATCYCTgCCTATgATgggTTT
Adenovirus	ADV2F-A	CCCMTTYAACCACCACCg	ADV1R-A	ACATCCTTBCKgAAgTTCCA
Enterovirus	5UTR-U447	TCCTCCggCCCCTgAATgCggCTAATCC	5UTR-L541	gAAACACggWCACCCAAAgTASTCg

10

Improvements in Multiplexing

15 Initially, multiplex detection of 7 respiratory pathogen targets at 500 copy sensitivity: RSV group A, RSV group B, Influenza A, HCoV-SARS, HCoV-229E, HCoV-OC43, and *M. pneumoniae* was determined. Subsequently, sensitivity was improved. Detection at 100 copy

sensitivity has been confirmed for 18 respiratory pathogen targets in a 20-plex assay (Table 8). Two of 20 targets, the influenza A M gene and influenza H1 gene, were detected at 500 copies. This typically corresponds in our laboratory to <0.001 $TCID_{50}$ per assay, a threshold comparable to many useful microbiological assays.

Table 8: Sensitivity of respiratory panel

	RSV A	RSV B	Influenza A (N1)	Influenza A (N2)	Influenza A (matrix)	Influenza A (H1)	Influenza A (H2)	Influenza A (H3)	Influenza A (H5)	Influenza B
500 copies	+	+	+	+	+	+	+	+	+	+
100 copies	+	+	+	+	-	-	+	+	+	+

	HCoV-SARS	HCoV-229E	HCoV-OC43	Metapneumo-virus (Eur.)	HPIV-1	HPIV-2	HPIV-3	M. pneumoniae	C. pneumoniae	L. pneumophilae
500 copies	+	+	+	+	+	+	+	+	+	+
100 copies	+	+	+	+	+	+	+	+	+	+

Clinical Samples

5   Although assays of synthetic targets were optimized in
a complex background of normal tissue nucleic acids,
analysis of clinical materials was performed. Banked
clinical respiratory specimens were obtained from
Cinnia Huang of the Wadsworth Laboratory of the New
10   York State Department of Health and Pilar Perez-Brena
of the National Center for Microbiology of Spain.
Organisms included: metapneumovirus (n=3), RSV-B (n=3),
RSV-A (n=2), adenovirus (n=2), HPIV-1 (n=1), HPIV-3
(n=2), HPIV-4 (n=2), enterovirus (n=2), SARS-CoV (n=4),
15   influenza A (n=2). Six representative results are shown
in Figure 18; Multiplex Mass Tag PCR analysis of six
human respiratory specimens. Signal to noise ratio is
on the ordinate and primer sets are listed on the
abscissa. Mass Tag primer sets employed in a single
20   tube assay are indicated at the bottom of the figure.
Fig. 18A - Influenza A (N1, M, H1) H1); 18B - Human
Parainfluenza Type 1; 18C - Respiratory Syncytial Group
B; 18D – Enterovirus; 18E – SARS CoV; and 18F - Human
Parainfluenza Type 3.

25

Pathogens

Tables 9-12 show a non-comprehenisve list of various
target pathogens and corresponding primer sequences. In
30   Table 10, the forward and reverse primer pairs for
Cytomegalovirus, SEQ ID NOS: 87 and 88; for HPIV-4A,
SEQ ID NOS: 37 and 38; for HPIV-4B, SEQ ID NOS: 39 and
40; for Measles, SEQ ID NOS: 91 and 92; for Varicella
Zoster virus, SEQ ID NOS: 89 and 90; for HIV-1, SEQ ID
35   NOS: 69 and 70; for HIV-2, SEQ ID NOS: 71 and 72; for
S. Pneumoniae, SEQ ID NOS: 100 and 101; for Haemophilus

Influenzae, SEQ ID NOS: 77 and 78; for Herpes Simplex, SEQ ID NOS: 67 and 68; for MV Canadian isolates, SEQ ID NOS: 29 and 30; for Adenovirus 2 A/B 505/630, SEQ ID NOS: 93 and 94; for Enterovirus A/B 702/495, SEQ ID
5   NOS: 95 and 96; and forward primers for Enterovirus A/B 702/495, SEQ ID NOS: 98 and 99.

10

Table 9

Primer sequence	Name	Target	Previous Masscode	Panel	
HIV2	HIV2TMFPR2	Forward A	588	Respiratory / Enc	30
HIV2	HIV2TMRPR2	Reverse B	570	Respiratory / Enc	
Streptococcus pneumoniae	SPPLY-U532	Forward A	714	Respiratory / Enc	31
Streptococcus pneumoniae	SPPLY-L606	Reverse B	694	Respiratory / Enc	
Haemophilus influenza	HINF-U82	Forward A	734	Respiratory / Enc	32
Haemophilus influenza	Hinf-L158	Reverse B	726	Respiratory / Enc	
Herpes Simplex	HSV-U27	Forward A	722	Respiratory / Enc	33
Herpes Simplex	HSV-L121	Reverse B	706	Respiratory / Enc	
Metaneumovirus Canadian	MV-Can-U918	Forward A	718	Respiratory	34
Metaneumovirus Canadian	MV-Can-L992	Reverse B	654	Respiratory	
Adenovirus	ADV2F-A	Forward A	503	Respiratory / Enc	12
Adenovirus	ADV1R-A	Reverse B	630	Respiratory / Enc	
Enterovirus	5UTR-U447	Forward A	702	Respiratory / Enc	14
Enterovirus	5UTR-U450	Forward A	702	Respiratory / Enc	
Enterovirus	5UTR-u457	Forward A	702	Respiratory / Enc	
Enterovirus	5UTR-L541	Reverse B	495	Respiratory / Enc	14
Neisseria meningitidis	Nmen-U829	Forward A	730	Encephalitis / Resp	
Neisseria meningitidis	Nmen-L892	Reverse B	439	Encephalitis / Resp	
WNV1	DF3 -87F	Forward A	539	Encephalitis	
WNV1	DF3 -156R	Reverse B	499	Encephalitis	
WNV2	WN-Ax-FWD	Forward A	539	Encephalitis	
WNV2	WN-Ax-REV	Reverse B	499	Encephalitis	
SLE	SLE-D-73F	Forward A	658	Encephalitis	
SLE	SLE-D-145R	Reverse B	642	Encephalitis	

Table 9. (Cont.)

Primer sequence	Name	Target	Previous Masscode	Panel	
Cytomegalovirus	CMV-U421	Forward A		Respiratory / Enc	24
Cytomegalovirus	CMV-L501	Reverse B		Respiratory / Enc	
HPIV4A	HPIV4A-U191	Forward A		Respiratory	25
HPIV4a	HPIV4A-L269	Reverse B		Respiratory	
HPIV4B	HPIV4B-U194	Forward A		Respiratory	26
HPIV4b	HPIV4B-L306	Reverse B		Respiratory	
Measles	MEA-U1103	Forward A		Respiratory / Enc	27
Measles	MEA-L1183	Reverse B		Respiratory / Enc	
VZV	VZV-U138	Forward A		Respiratory / Enc	28
VZV	VZV-L196	Reverse B		Respiratory / Enc	
HIV1	SK68i	Forward A		Respiratory / Enc	29
HIV1	SK69i	Reverse B	383	Respiratory / Enc	

Table 9 (Cont.)

Primer sequence	Name	Target	Previous Masscode	Panel	
RSV A gen N	RSA-U1137	Forward A	467	Respiratory	1
RSV A gen N	RSV-L1192	Reverse B	455	Respiratory	
RSV B gen N	RSB-U1248	Forward A	483	Respiratory	2
RSV B gen N	RSV-1318	Reverse B	479	Respiratory	
Flu A - N1	NA1-U1078	Forward A	499	Respiratory	3
Flu A - N1	NA1-L1352	Reverse B	439	Respiratory	
Flu A - N2	NA2-U560	Forward A	658	Respiratory	4
Flu A - N2	NA2-L858	Reverse B	730	Respiratory	
Flu A (MATRIX)	AM-U151	Forward A	618	Respiratory / Enc	5
Flu A (MATRIX)	AM-L397	Reverse B	680	Respiratory / Enc	
Flu B	BHA-U188	Forward A	698	Respiratory / Enc	6
Flu B	BHA-L347	Reverse B	598	Respiratory / Enc	
SARS-Coronavirus	CIID-28891F	Forward A	527	Respiratory / Enc	7
SARS-Coronavirus	CIID-29100R	Reverse B	666	Respiratory / Enc	
229E-Coronavirus	Taq-Co22-418F	ForwardA	670	Respiratory / Enc	8
229E-Coronavirus	Taq-Co22-636R	Reverse B	558	Respiratory / Enc	
OC43-Coronavirus	Taq-Co43-270F	ForwardA	686	Respiratory / Enc	9
OC43-Coronavirus	Taq-Co43-508R	Reverse B	548	Respiratory / Enc	
Metapneumovirus	MPV01.2	ForwardA	718	Respiratory	10
Metapneumovirus	MPV02.2	Reverse B	654	Respiratory	
Mycoplasma pneumoniae	MTPM1	Forward A	602	Respiratory	11
Mycoplasma pneumoniae	MTPM2	Reverse B	814	Respiratory	

Table 9 (Cont.)

Primer sequence	Name	Target	Previous Masscode	Panel	
adenovirus	ADV1F-A	Forward A	503	Respiratory / Enc	12
adenovirus	ADV2R-A	Reverse B	830	Respiratory / Enc	
Chlamydia	CLPM1	Forward A	519	Respiratory	13
Chlamydia	CLPM2	Reverse B	971	Respiratory	
enterovirus	EV1f	Forward A	702	Respiratory / Enc	14
enterovirus	EV1r	Reverse B	495	Respiratory / Enc	
flavivirus1	Fla-U8093	Forward A	710	Encephalitis	15
flavivirus1	Fla-L9279	Reverse B	594	Encephalitis	
flavivirus2	Fla-U8954	Forward A	710	Encephalitis	15
flavivirus2	Fla-L10098	Reverse B	594	Encephalitis	
fluHA1	HA1-U583	Forward A	850	Respiratory	16
fluHA1	HA1-L895	Reverse B	634	Respiratory	
fluHA2	H2A208U27	Forward A	662	Respiratory	17
fluHA2	H2A559L26	Reverse B	630	Respiratory	
fluHA3	HA3-U115	Forward A	375	Respiratory	18
fluHA3	HA3-L380	Reverse B	475	Respiratory	
fluHA5	HA5-u71	Forward A	646	Respiratory	19
fluHA5	HA5-L147	Reverse B	395	Respiratory	
HPIV1	HPIV1-U62	Forward A	566	Respiratory	20
HPIV1	HPIV1-L167	Reverse B	357	Respiratory	
HPIV2	HPIV2-U908	Forward A	483	Respiratory	21
HPIV2	HPIV2-L984	Reverse B	590	Respiratory	
HPIV3	HPIV3-U590	Forward A	642	Respiratory	22
HPIV3	HPIV3-L668	Reverse B	539	Respiratory	
Legionella1	Legpneu-U149	Forward A	678	Respiratory	23
Legionella1	LegPneu-L223	Reverse B	582	Respiratory	

Table 10

Respiratory Panel Mass-Tag Primers

Tagged Pairs	Tier	Standards	Primer Name	Start	Length	Tm	Primer forward	Primer Name	Start	Length	Tm	Primer reverse	reference
CYTOMEGALOVIRUS	1	YES	CMV-U421	421	25	64.57	TACAGCACGTCAACCAACCCT	CMV-L501	501	25	65.08	CCC GGC CTT CAC CAA CCG AAA A	GIDL
HPIV-4A	1		HPIV4A-U191	191	24	59	AACAGAAGGAAATGATGGTGGAAC	HPIV4A-L269	269	20	59	TGCTGTGGATGTATGGGCAG	GIDL
HPIV-4B	1	cloning	HPIV4B-U194	194	25	59	AGAAGAAAGCAACGATGAGAGCAAGG	HPIV4B-L306	306	23	59	GTTCCTGGTTCACTCTCTTCA	GIDL
MEASLES	1	synthetic	MEA-U1103	1100	25	59.33	CAAGCATCATGATTYGCATTCCTGG	MEA-L1183	1183	26	59.98	GCT GAA TCT CTG CCT ATG ATG GGT TT	GIDL
VARICELLA ZOSTER VIRUS	1	YES	VZV-U138	138	23	59.84	ACGTGGATCGTCGGATCAGTTGT	VZV-L196	196	23	59.97	TCG CTA TGT GCT AAA ACA CGC GG	GIDL
HIV1	1	Thomas	SK68	SK68	28	70 to 75	TTC TTT GGA GGA GGC GGA AGC AGA TGG	SK69	SK69	28		TTAATCCCCAGGAGCTAGTTCAACA	GIDL
HIV2	1	synthetic	HIV2TMFPR2	hiv2tmfpr2	18		GGCTGCACGCCCTATGATA	HIV2TMRPR2				TCTGCATGGCTGCCTTGATG	H Eberpreis
STREPTOCOCCUS PNEUMONIAE	1	synthetic	SPPLY-U532	532	22	59	ACCGATAGCTTTCTCCAAGTGG	SPPLY-L606	606	23	59	CTAGCCAACAAATGCGTTACCG	GIDL
HAEMOPHILUS INFLUENZAE	1	synthetic	HINF-U82	82	27	59	AAGCTCCTTGAATTTTTGTATTAGAA	Hinf-L158	158	23	59	GCTGAATTGGCTTGGATAGCGAG	GIDL
HERPES SIMPLEX	1	YES	HSV-U27	27	24	62.09	CCCGGATGCCGTCCAGAGCAATAT	HSV-L121	121	24	61.55	CCC GCG GAG GTT GTA CAA AAA GCT	GIDL
MV-Canadian isolates	1	synthetic	MV-Can-U918	918	24	59	AAGTCCAAAGGCAGGRCTGTTATC	MV-Can-L992	992	25	60	CCTGAAGCATTCCAAGAACAAC	GIDL
Adenovirus2 A/B 503630	1	YES	ADV2F-A	ADV2F-A		59.10 to 81	CCAHTTYAACCACCACCG	ADVIR-A	ADVIR-A	25	54.10 to 59	ACATCCTTGCKGAAGTCTTCCA	Ana Aveson
Enterovirus A/B 702/495	1	YES	5UIR-U447	447		76	TCCTCCGGCCCCTGAATGCGGCTAATCC						
Enterovirus A/B 702/495	1	YES	5UIR-U450	450		72	TCCGGCCCTGAATGCGGCTAATCC						
Enterovirus A/B 702/495	1	YES	5UIR-U457	457		63	CCCTGAATGCGGCTAATCC	5UIR-L541	5UIR-L541	28	87 to 81	GAAACACGGWCACCAAAGTASTCG	Ana Aveson

References: Schneider, M. Virol Res (2000) 113-120; H Eberpreis, Robert Koch Institute; Ana Aveson J Vir 82 (2001) 113-120

Table 11

Targeted Pairs	Standards	LIST OF PRIMERS	Name FWD	Forward - A	Tm primer	Name REV	Reverse - B	Tm primer	Product Size
RSVA - 1 A/B 467/455	YES	RSV A gen N	RSA-U1137	AGATCAACTTCTGTCATCCACCAA	62	RSV-L1192	GCACATCATAATTAGGAGTATCAAT	56	80
		RSV A gen N	rsh1ca-L2-777F	GGTGCAGGGCAAGTGATGTTA	63	rsh1ca-L2-1013R	GCCAGCAGCCATGCCTAATAC	62	240
		RSV A gen P	RSHP1-L2-235F	CAGGGAACAAGCCGAATTATCA	63	RSHP1-L2-540R	CTCTTAAACCAACCATGCCATCTC	63	320
RSVB - 1 A/B 483/479	YES	RSV B gen N	RSB-U1246	AAGATGCAAATCATAAATTCACAGGA	62	RSV-L1318	TGATATCCAGCATCTTTAAGTATCTTTATAGTG	62	105
	YES	RSV B gen N	rshbcomp-L2-775F	ATGGTTCAGGGCCAAGTAATGCT	63	rshcomp-L2-913R	TCTCCTCCCAACTTCTGTGCA	63	180
	YES	RSV B gen P	RSHPQ-L2-189F	TCTGGCCAACATCATCAATC	63	RSHPQ-L2-295R	GGGGTGAGATCTTCTTTGAAGCT	62	120
FluA-N1 A/B 499/439	YES	N1	NA1-U1078	ATGGTAATGGTGTTTGGATAGGAAG	61	NA1-L1352	AATGGTCGTCCCCACTAGTCCAG	62	274
FluA-N2 A/B 650/730	YES	N2	NA2-U560	AAGCATGGCTGCATGTTTGTG	64	NA2-L858	AGCAGGATATCGGAGGATAACAGGA	62	298
FluA-M A/B 618/690	YES	A (MATRIX)	AM-U151	CATGGAATGGCTAAAGACAAGACC	63	AM-L397	AAGTGCACCAGCAGAATAACTGAG	62	246
FluB A/B 698/598	YES	B	BHA-U188	AGAACCAGAGGGGAAACTATGCCC	63	BHA-L347	CTGTCGTGCATATAGGGAAAGCAG	62	159
SARS - A/B 527/666	YES	SARS-Coronavirus	CIID-20891F	AAgCCTCgCCAAAAACgTAC	62	CIID-29100R	AAgTCAgCCATgTTCCCgAA	63	130
228E A/B 670/558	YES	228E-Coronavirus	Taq-Co22-416F	ggCgCAAgAATTCAgAACCA	64	Taq-Co22-636R	TAApAgCCgCAgCAACTgC	63	240
OC43 A/B 686/548	YES	OC43-Coronavirus	Taq-Co43-270F	TgTgCCTATTgCACCAggAgT	63	Taq-Co43-508R	CCCgATCgACAATgTCAgC	63	260
Metapnuemo A/B 718/654	YES	Metapneumovirus	MPV01.2	AACCGTGTACTAAGTGATGCACTC	60	MPV02.2	CATTGTTTGACCGGCCCCATAA	60	205
Mycoplasma - 1 A/B 602/614	YES	Mycoplasma1	MTPM1	CCAACCAAACAACAACGTTCA	62	MTPM2	ACCTTGACTGGAGGCCCGTTA	62	76
	YES	Mycoplasma2	MpnA	CCGCCGAGCAATGAAAAACTCC	60	MpnB	TCGAGCCCGATCATTTGGGAGGT	63	360
HPV1 A/B 566/357	YES	Parainfluenza 1	HPV1-U82	TACTTTGACACATTAGTTCCAGGAG	61	HPV1-L167	CGGTACTTCTTTGACCAGGTATAATG	62	110
HPV2 A/B 566/357	YES	Parainfluenza 2	HPV2-U908	GGACTTCGGAACAAGATGCCCT	63	HPV2-L984	AGCATGGAGCCYTTAATTTCTGGA	63	102
HPV3 A/B 566/357	YES	Parainfluenza 3	HPV3-U590	CCTTTCAGACAACGATGGAACAGTG	62	HPV3-L668	GCATKATTGACCCAATCTGATCC	63	103
Legionella 1 A/B 678/582	YES	Legionella1	Legpneu-U149	GCATWGATGTTARTCCCGAAGCA	66	Legpneu-L223	CGGTAAAGCCAATTGAGCG	63	79
	YES	Legionella2	LGPM1	AAAGGCATGCAAGACGCCTATG	63	LGPM2	TGTTAACAACGTCTTTCATTTGCTG	62	75
	YES	Legionella3	LpnA	GGCGGACTATAGCCATTTGGAA	56	LpnB	GCCATGACCTACTTTCCCATGA	58	100
Chlamydia A/B 519/383	YES	Chlamydia pneumoniae	CLPM1	CATGGTGTCATTCGCCAAGT	62	CLPM2	CGTGTCGTCCAGCCATTTTA	62	85
FluHA1 A/B 650/590	YES	HA1	HA1-U583	GGTGTTCATCACCGCTCTAACAT	62	HA1-L895	GTGTTTGCACTTGCGCGTCACAT	65	312
FluHA2 A/B 662/539	YES	HA2	H2A208U27	GCTATGCAAACTAAACGGAATYCCTCC	67	H2A559L26	TATTGTTGTACGGATCCTTTGGCAACC	66	377
FluHA3-1 A/B 586/475	YES	HA3	HA3-U115	GCTACTGAGCCTGGTTCAGAGTTC	60	HA3-L375	GAAGTCTTCATTGATAAACTCCAG	56	260
FluHA3-2 A/B 586/475	YES	HA3	HA3-U115	CCTACTGAGCCTGGTTCAGAGTTC	60	HA3-L380	ATGCTGAGCGACTCCAGTCC	60	265
FluHAS A/B 646/395	YES	HAS-human	HAShuman-u71	TTACTGTTACACATGCCCAAGACA	62	HAShuman-L147	AGGyTTCACTCCATTTAGATCGCA	64	105

Table 12

Primer sequence		Name	Target	Previous Masscode	Panel
TACAGCACGCTCAACACCAACGCCT	25	CMV-U421	Citomegalovirus		Respiratory
AACAGAAGGAAATGATGGTGGAAC	24	HPIV4A-U191	HPIV4A		Respiratory
AGAAGAAAACAACGATGAGACAAGG	25	HPIV4B-U194	HPIV4B		Respiratory
CAAGCATCATGATYGCCATTCCTGG	25	MEA-U1103	Measles		Respiratory
ACGTGGATCGTCGGATCAGTTGT	23	VZV-U138	VZV		Respiratory
TTCTTGGAGCAGCIGGAAGCACIATGG	28	SK68i	HIV1		Respiratory
GGCTGCACGCCCTATGATA	19	HIV2TMFPR2	HIV2		Respiratory
AGCGATAGCTTTCTCCAAGTGG	22	SPPLY-U532	Streptococcus pneumonie		Respiratory
AAGCTCCTTGMATTTTTTGTATTAGAA	27	HINF-U82	Haemophilus Influenza		Respiratory
CCCGGATGCGGTCCAGACGATTAT	24	HSV-U27	Herpes Simplex		Respiratory
AAGTCCAAAGGCAGGRCTGTTATC	24	MV-Can-U918	Metaneumovirus Canadian		Respiratory
CCCMTTYAACCACCACCG	18	ADV2F-A	Adenovirus	Adenovirus2_503	Respiratory
TCCTCCGGCCCCTGAATGCGGCTAATCC	28	5UTR-U447	Enterovirus	EnteroVirus_702	Respiratory
TCCGGCCCCTGAATGCGGCTAATCC	25	5UTR-U450	Enterovirus	EnteroVirus_702	Respiratory
CCCCTGAATGCGGCTAATCC	20	5UTR-u457	Enterovirus	EnteroVirus_702	Respiratory
CCCGGCCTTCACCACCAACCGAAAA	25	CMV-L501	Citomegalovirus		Respiratory
TGCTGTGGATGTATGGGCAG	20	HPIV4A-L269	HPIV4a		Respiratory
GTTTCCCTGGTTCACTCTCTTCA	23	HPIV4B-L306	HPIV4b		Respiratory
CCTGAATCYCTGCCTATGATGGGTTT	26	MEA-L1183	Measles		Respiratory
TCGCTATGTGCTAAAACACGCGG	23	VZV-L196	VZV		Respiratory
TTMATGCCCCAGACIGTIAGTTICAACA	28	SK69i	HIV1		Respiratory
TCTGCATGGCTGCTTGATG	19	HIV2TMRPR2	HIV2		Respiratory
CTTAGCCAACAAATCGTTTACCG	23	SPPLY-L606	Streptococcus pneumonie		Respiratory
GCTGAATTGGCTTRGATACCGAG	23	Hinf-L158	Haemophilus influenza		Respiratory
CCCGCGGAGGTTGTACAAAAAGCT	24	HSV-L121	Herpes Simplex		Respiratory
CCTGAAGCATTRCCAAGAACAACAC	25	MV-Can-L992	Metaneumovirus Canadian		Respiratory
ACATCCTTBCKGAAGTTCCA	20	ADV1R-A	Adenovirus	Adenovirus2_630	Respiratory
GAAACACGGWCACCCAAAGTASTCG	25	5UTR-L541	Enterovirus	EnteroVirus_495	Respiratory
AACACCGGGTCTTAATTCTTATATCAA	27	EboZp-U234	Ebola Zaire		Hemorrhagic Fevers
TTCCGTCACAAGCCGAAATT	20	Mar-U292	Marburg		Hemorrhagic Fevers
AGAACACGTGCCGCTTACGCCCA	23	CCHV-U4	CCHV		Hemorrhagic Fevers
TCCCAAAGATGTTAGTGCCTGA	22	Sabia-U344	Sabia		Hemorrhagic Fevers
CCACCCGTCACCTGAGAGACACAATT	26	Machupo-U212	Machupo		Hemorrhagic Fevers
GCTGGGAGCGCGGGTATC	17	YF-U186	Yellow Fever		Hemorrhagic Fevers
GGATTGACCTGTGCCTGTTGC	21	RVF-U578	Rift Valley fever		Hemorrhagic Fevers
TCTGAAGCCATTGGCCGT	18	Nmen-U829	Neisseria meningitidis		Hemorrhagic Fevers
CRTATTATTAMTGGCTATAAATGTTGC	27	RSF-U255	Rickettsia Spotted fever		Hemorrhagic Fevers
YACAATGACMGATGAGGTTGTRGC	24	Bburg-U896	Borrelia burgdorferi		Hemorrhagic Fevers
GATGGAGGRTGCATCATGG	19	OMSK-U171	OMSK		Hemorrhagic Fevers
AACTTAGGAGCTACCCAAAACAGC	24	CHKP-U68	Chikungunya POL		Hemorrhagic Fevers
CAATGTCYTCMGCCTGGACACCT	23	CHKE-U223	Chikungunya ENV		Hemorrhagic Fevers
AYACAGCAGCAGTTAGCCTCCT	22	HAN-U179	Hantaan		Hemorrhagic Fevers
ATGAARGCAGATGARATYACACC	23	OOB-U222	Dobrava		Hemorrhagic Fevers
AAGGTGTTTTGATCAGGCTAGAGA	25	TAC-U114	Tacaribe		Hemorrhagic Fevers
GCCRTGTGARTGCCTRCTTCCATT	24	GUAV-U321	Guananto		Hemorrhagic Fevers
CAGGATTGCAGCAGGGAAGA	20	SEO-U243	Seoul		Hemorrhagic Fevers
TGGAAGCCTGGCTGAAAGAG	20	KYF-U170	Kyasanur forest		Hemorrhagic Fevers
TGACCTTYACMAATGAYTCCAY	22	LCMV-U47b	LCMV		Hemorrhagic Fevers
GGTGGTAAAATTCCCATAGTAGTTCTTT	28	EboZA-L319	Ebola Zaire		Hemorrhagic Fevers
TTATTTTAGTTGAGAAAAGAGGTTCATGC	29	Mar-L372	Marburg		Hemorrhagic Fevers
CCATTCYTTYTTRAACTCYTCAAACCA	27	CCHV-L120	CCHV		Hemorrhagic Fevers
CCTGCACTGACAATCGCTTG	20	SABIA-L424	Sabia		Hemorrhagic Fevers
TGCAAGTCAAGCGAAAAGAGGGGATG	26	Machupo-L290	Machupo		Hemorrhagic Fevers
GGAAGCCCAATGGTCCTCAT	20	YF-L249	Yellow Fever		Hemorrhagic Fevers
GCATTAGAAATGTCCTCTTTTGCTGC	26	RVF-L660	Rift Valley fever		Hemorrhagic Fevers
CAAACACACCACGCGCAT	18	Nmen-L892	Neisseria meningitidis		Hemorrhagic Fevers
ACKRTTTAAAGTTAARCTTTTGCC	24	RSF-L394	Rickettsia Spotted fever		Hemorrhagic Fevers
GCAATGACAAAACATATTGRGGAASTTGA	29	Bburg-L977	Borrelia burgdorferi		Hemorrhagic Fevers
TGACCACTTGGCCTGATCC	19	OMSK-L234	OMSK		Hemorrhagic Fevers
GGACGGTACAGCGCTTCTG	19	CHKP-L132	Chikungunya POL		Hemorrhagic Fevers
TCRCCAAATTGTCCTGGTCTTCCTG	25	CHKE-L310	Chikungunya ENV		Hemorrhagic Fevers
GCTGCCGTARGTAGTCCCTGTT	22	HAN-L245	Hantaan		Hemorrhagic Fevers
CCTGRGCTGGRTATARTCCACA	22	OOB-L289	Dobrava		Hemorrhagic Fevers
CCATCCTTGATGGTGGTAACATG	23	TAC-L192	Tacaribe		Hemorrhagic Fevers
TATGTRCACTGYTTCAGAAAACCTCA	26	GUA-L265	Guanarito		Hemorrhagic Fevers
ATGATCACCAGGYTCTACCCC	21	SEOUL-L309	Seoul		Hemorrhagic Fevers
TCATCCCCACTGACCAGCAT	20	KYF-L233	Kypsanur forest		Hemorrhagic Fevers
TATRCTCATGAGTGTGTGGTCAA	23	LCMV-L142a	LCMV	Same than below	Hemorrhagic Fevers
TATRCTCATAAGTGTGTGATCAA	23	LCMV-L142b	LCMV	Same than above	Hemorrhagic Fevers
	1598				

Example 7

Efficient laboratory diagnosis of infectious diseases is
increasingly important to clinical management and public
health. Methods to directly detect nucleic acids of
microbial pathogens in clinical specimens are rapid,
sensitive, and may succeed when culturing the organism
fails. Clinical syndromes are infrequently specific for
single pathogens; thus, assays are needed that allow
multiple agents to be simultaneously considered. Current
multiplex assays employ gel-based formats in which
products are distinguished by size, fluorescent reporter
dyes that vary in color, or secondary enzyme
hybridization assays. Gel-based assays are reported that
detect 2-8 different targets with sensitivities of 2-100
PFU or less than 1-5 PFU, depending on whether
amplification is carried out in a single or nested
format, respectively (1-4). Fluorescence reporter
systems achieve quantitative detection with sensitivity
similar to that of nested amplification; however, their
capacity to simultaneously query multiple targets is
limited to the number of fluorescent emission peaks that
can be unequivocally resolved. At present, up to 4
fluorescent reporter dyes can be detected simultaneously
(5,6). Multiplex detection of up to 9 pathogens has been
achieved in hybridization enzyme systems; however, the
method requires cumbersome postamplification processing
(7).

Experimental Results

To address the need for sensitive multiplex assays in
diagnostic molecular microbiology, we created a
polymerase chain reaction (PCR) platform in which
microbial gene targets are coded by a library of 64
distinct Masscode tags (Qiagen Masscode technology,
Qiagen, Hilden, Germany). A schematic representation of
this approach is shown in Figure 22. Microbial nucleic
acids (RNA, DNA, or both) are amplified by multiplex
reverse transcription (RT)-PCR using primers labeled by
a photocleavable link to molecular tags of different
molecular weight. After removing unincorporated primers,
tags are released by UV irradiation and analyzed by mass
spectrometry. The identity of the microbe in the
clinical sample is determined by its cognate tags. As a
first test of this technology, we focused on respiratory
disease because differential diagnosis is a common
clinical challenge, with implications for outbreak
control and individual case management. Multiplex primer
sets were designed to identify up to 22 respiratory
pathogens in a single Mass Tag PCR reaction; sensitivity
was established by using synthetic DNA and RNA standards
as well as titered viral stocks; the utility of Mass Tag
PCR was determined in blinded analysis of previously
diagnosed clinical specimens. Oligonucleotide primers
were designed in conserved genomic regions to detect the
broadest number of members for a given pathogen species
by efficiently amplifying a 50- to 300-bp product. In
some instances, we selected established primer sets; in
others, we used a software program designed to cull
sequence information from GenBank, perform multiple

alignments, and maximize multiplex performance by selecting primers with uniform melting temperatures and minimal cross-hybridization potential (Appendix Table, available at http://www.cdc.

5   gov/ncidod/eid/vol11no02/04-0492_app.htm). Primers, synthesized with a 5'C6 spacer and aminohexyl modification, were covalently conjugated by a photocleavable link to Masscode tags (Qiagen Masscode technology) (8,9). Masscode tags have a modular

0   structure, including a tetrafluorophenyl ester for tag conjugation to primary amines; an o-nitrobenzyl photolabile linker for photoredox cleavage of the tag from the analyte; a mass spectrometry sensitivity enhancer, which improves the efficiency of atmospheric

5   pressure chemical ionization of the cleaved tag; and a variable mass unit for variation of the cleaved tag mass (8,10-12). A library of 64 different tags has been established. Forward and reverse primers in individual primer sets are labeled with distinct molecular weight

0   tags. Thus, amplification of a microbial gene target produces a dual signal that allows assessment of specificity. Gene target standards were cloned by PCR into pCR2.1-TOPO (Invitrogen, Carlsbad, CA, USA) by using DNA template (bacterial and DNA viral targets) or

5   cDNA template (RNA viral targets) obtained by reverse transcription of extracts from infected cultured cells or by assembly of overlapping synthetic polynucleotides. Assays were initially established by using plasmid standards diluted in 2.5-μg/mL human placenta DNA

)   (Sigma, St. Louis, MO, USA) and subjected to PCR amplification with a multiplex PCR kit (Qiagen), primers at 0.5 μmol/L each, and the following cycling protocol:

an annealing step with a temperature reduction in 1°C increments from 65°C to 51°C during the first 15 cycles and then continuing with a cycling profile of 94°C for 20 s, 50°C for 20 s, and 72°C for 30 s in an MJ PTC200

5   thermal cycler (MJ Research, Waltham, MA, USA). Amplification products were separated from unused primers by using QIAquick 96 PCR purification cartridges (Qiagen, with modified binding and wash buffers). Masscode tags were decoupled from amplified products

0   through UV light-induced photolysis in a flow cell and analyzed in a single quadrapole mass spectrometer using positive-mode atmospheric pressure chemical ionization (Agilent Technologies, Palo Alto, CA, USA). A detection threshold of 100 DNA copies was determined for 19 of 22

5   cloned targets by using a 22-plex assay (Table 1). Many respiratory pathogens have RNA genomes; thus, where indicated, assay sensitivity was determined by using synthetic RNA standards or RNA extracts of viral stocks. Synthetic RNA standards were generated by using T7

0   polymerase and linearized plasmid DNA. After quantitation by UV spectrometry, RNA was serially diluted in 2.5-μg/mL yeast tRNA (Sigma), reverse transcribed with random hexamers by using Superscript II (Invitrogen, Carlsbad, CA, USA), and used as template

5   for Mass Tag PCR. As anticipated, sensitivity was reduced by the use of RNA instead of DNA templates (Table 15).

0

Table 15

Pathogen or protein	Detection threshold (DNA copies/RNA copies)
Influenza A matrix	100/1,000
Influenza A N1	100/NA
Influenza A N2	100/NA
Influenza A H1	100/NA
Influenza A H2	100/NA
Influenza A H3	100/NA
Influenza A H5	100/NA
Influenza B H	500/1,000
RSV group A	100/1,000
RSV group B	100/500
Metapneumovirus	100/1,000
CoV-SARS	100/500
CoV-OC43	100/500
CoV-229E	100/500
HPIV-1	100/1,000
HPIV-2	100/1,000
HPIV-3	100/500
Chlamydia pneumoniae	100/NA
Mycoplasma pneumoniae	100/NA
Legionella pneumophila	100/NA
Enterovirus (genus)	500/1,000
Adenovirus (genus)	5,000/NA

*NA, not assessed; RSV, respiratory syncytial virus; CoV, coronavirus; SARS, severe acute respiratory syndrome; HPIV, human parainfluenza virus.

The sensitivity of Mass Tag PCR to detect live virus was tested by using RNA extracted from serial dilutions of titered stocks of coronaviruses (severe acute respiratory syndrome [SARS] and OC43) and parainfluenzaviruses (HPIV 2 and 3). A 100-μL volume of each dilution was analyzed. RNA extracted from a 1-TCID50/mL dilution, representing 0.025 TCID50 per PCR reaction, was consistently positive in Mass Tag PCR. RNA extracted from banked sputum, nasal swabs, and pulmonary washes of persons with respiratory infection was tested by using an assay panel comprising 30 gene targets that

represented 22 respiratory pathogens. Infection in each
of these persons had been previously diagnosed through
virus isolation, conventional nested RT-PCR, or both.
Reverse transcription was performed using random
5   hexamers, and Mass Tag PCR results were consistent in
all cases with the established diagnosis. Infections
with respiratory syncytial virus, human parainfluenza
virus, SARS coronavirus, adenovirus, enterovirus,
metapneumovirus, and influenza virus were correctly
0   identified (Table 16 and Figure 23).

Table 16

Pathogen	No. positive/no. tested†
RSV A	2/2
RSV B	3/3
HPIV-1	1/1
HPIV-3	2/2
HPIV-4	2/2
CoV-SARS	4/4
Metapneumovirus	2/3
Influenza B	1/3
Influenza A	2/6
Adenovirus	2/2
Enterovirus	2/2

*RSV, respiratory syncytial virus; HPIV, human parainfluenza virus; CoV,
coronavirus; SARS, severe acute respiratory syndrome.
†No. positive and consistent with previous diagnosis/number tested (with
respective previous diagnosis).

5

A panel comprising gene targets representing 17
pathogens related to central nervous system infectious
0   disease (influenza A virus matrix gene; influenza B
virus; human coronaviruses 229E, OC43, and SARS;
enterovirus; adenovirus; human herpesvirus-1 and -3;
West Nile virus; St. Louis encephalitis virus; measles
virus; HIV-1 and -2; and Streptococcus pneumoniae,

Haemophilus influenzae, and *Nesseria meningitidis*) was applied to RNA obtained from banked samples of cerebrospinal fluid and brain tissue that had been previously characterized by conventional diagnostic RT-PCR. Two of 3 cases of West Nile virus encephalitis were correctly identified. Eleven of 12 cases of enteroviral meningitis were detected representing serotypes CV-B2, CV-B3, CV-B5, E-6, E-11, E-13, E-18, and E-30 (data not shown).

Conclusions

Our results indicate that Mass Tag PCR is a sensitive and specific tool for molecular characterization of microflora. The advantage of Mass Tag PCR is its capacity for multiplex analysis. Although the use of degenerate primers (e.g., enteroviruses and adenoviruses, and Table 16) may reduce sensitivity, the limit of multiplexing to detect specific targets will likely be defined by the maximal primer concentration that can be accommodated in a PCR mix. Analysis requires the purification of product from unincorporated primers and mass spectroscopy. Although these steps are now performed manually, and mass spectrometers are not yet widely distributed in clinical laboratories, the increasing popularity of mass spectrometry in biomedical sciences and the advent of smaller, lower-cost instruments could facilitate wider use additional pathogen panels, our continuing work is focused on optimizing multiplexing, sensitivity, and throughput. Potential applications include differential diagnosis of

infectious   diseases,   blood   product   surveillance,
forensic microbiology, and biodefense.

What is claimed is:

1.   A method for simultaneously detecting in a sample
     the presence of one or more of a plurality of
     different target nucleic acids comprising the steps
     of:
     (a)   contacting the sample with a plurality of
           nucleic acid primers simultaneously and under
           conditions permitting, and for a time
           sufficient for, primer extension to occur,
           wherein (i) for each target nucleic acid at
           least one predetermined primer is used which is
           specific for that target nucleic acid, (ii)
           each primer has a mass tag of predetermined
           size bound thereto via a labile bond, and (iii)
           the mass tag bound to any primer specific
           for one target nucleic acid has a different
           mass than the mass tag bound to any primer
           specific for any other target nucleic acid;
     (b)   separating any unextended primers from any
           extended primers;
     (c)   simultaneously cleaving the mass tags from any
           extended primers; and
     (d)   simultaneously determining the presence and
           sizes of any mass tags so cleaved,
     wherein the presence of a cleaved mass tag having
     the same size as a mass tag of predetermined size
     previously bound to a predetermined primer indicates
     the presence in the sample of the target nucleic
     acid specifically recognized by that predetermined
     primer.

2.   The method of claim 1, wherein the method detects
       the presence in the sample of 10 or more different
       target nucleic acids.

3.   The method of claim 1, wherein the method detects
       the presence in the sample of 50 or more different
       target nucleic acids.

4.   The method of claim 1, wherein the method detects
       the presence in the sample of 100 or more different
       target nucleic acids.

5.   The method of claim 1, wherein the method detects
       the presence in the sample of 200 or more different
       target nucleic acids.

6.   The method of claim 1, wherein the sample is
       contacted with 4 or more different primers.

7.   The method of claim 1, wherein the sample is
       contacted with 10 or more different primers.

8.   The method of claim 1, wherein the sample is
       contacted with 50 or more different primers.

9.   The method of claim 1, wherein the sample is
       contacted with 100 or more different primers.

10.   The method of claim 1, wherein the sample is
   contacted with 200 or more different primers.

11. The method of claim 1, wherein one or more primers comprises the sequence set forth in one of SEQ ID NOs:1-96.

12. The method of claim 1, wherein at least two different primers are specific for the same target nucleic acid.

13. The method of claim 12, wherein a first primer is a forward primer for the target nucleic acid and a second primer is a reverse primer for the same target nucleic acid.

14. The method of claim 13, wherein the mass tags bound to the first and second primers are of the same size.

15. The method of claim 13, wherein the mass tags bound to the first and second primers are of a different size.

16. The method of claim 12, wherein a first primer is directed to a 5'-UTR of the target nucleic acid and a second primer is directed to a 3D polymerase region of the target nucleic acid.

17. The method of claim 1, wherein each primer is from 15 to 30 nucleotides in length.

18. The method of claim 1, wherein each mass tag has a molecular weight of from 100Da to 2,500Da.

19. The method of claim 1, wherein the labile bond is a
     photolabile bond.

20. The method of claim 19, wherein the photolabile bond
     is cleavable by ultraviolet light.

21. The method of claim 1, wherein at least one target
     nucleic acid is from a pathogen.

22. The method of claim 21, wherein the pathogen is
     selected from the group consisting of B. anthracis,
     a Dengue virus, a West Nile virus, Japanese
     encephalitis virus, St. Louis encephalitis virus,
     Yellow Fever virus, La Crosse virus, California
     encephalitis virus, Rift Valley Fever virus, CCHF
     virus, VEE virus, EEE virus, WEE virus, Ebola virus,
     Marburg virus, LCMV, Junin virus, Machupo virus,
     Variola virus, SARS corona virus, an enterovirus, an
     influenza virus, a parainfluenza virus, a
     respiratory syncytial virus, a bunyavirus, a
     flavivirus, and an alphavirus.

23. The method of claim 21, wherein the pathogen is a
     respiratory pathogen.

24. The method of claim 23, wherein the respiratory
     pathogen is selected from the group consisting of
     respiratory syncytial virus A, respiratory syncytial
     virus B, Influenza A (N1), Influenza A (N2),
     Influenza A (M), Influenza A (H1), Influenza A (H2),
     Influenza A (H3), Influenza A (H5), Influenza B,
     SARS coronavirus, 229E coronavirus, OC43
     coronavirus, Metapneumovirus European,

Metapneumovirus   Canadian,   Parainfluenza   1,
Parainfluenza 2, Parainfluenza 3, Parainfluenza 4A,
Parainfluenza 4B, Cytomegalovirus, Measles virus,
Adenovirus,   Enterovirus,   M.   pneumoniae,   L.
pneumophilae, and C. pneumoniae.

25. The method of claim 21, wherein the pathogen is an
     encephalitis-inducing pathogen.

26. The method of claim 25, wherein the encephalitis-
     inducing pathogen is selected from the group
     consisting of West Nile virus, St. Louis
     encephalitis virus, Herpes Simplex virus, HIV 1, HIV
     2, N. meningitides, S. pneumoniae, H. influenzae,
     Influenza B, SARS coronavirus, 229E-CoV, OC43-CoV,
     Cytomegalovirus, and a Varicella Zoster virus.

27. The method of claim 21, wherein the pathogen is a
     hemorrhagic fever-inducing pathogen.

28. The method of claim 1, wherein the sample is a
     forensic sample.

29. The method of claim 1, wherein the sample is a food
     sample.

30. The method of claim 1, wherein the sample is blood,
     or a derivative of blood.

31. The method of claim 1, wherein the sample is a
     biological warfare agent or a suspected biological
     warfare agent.

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32. The method of claim 1, wherein the mass tag is selected from the group consisting of:
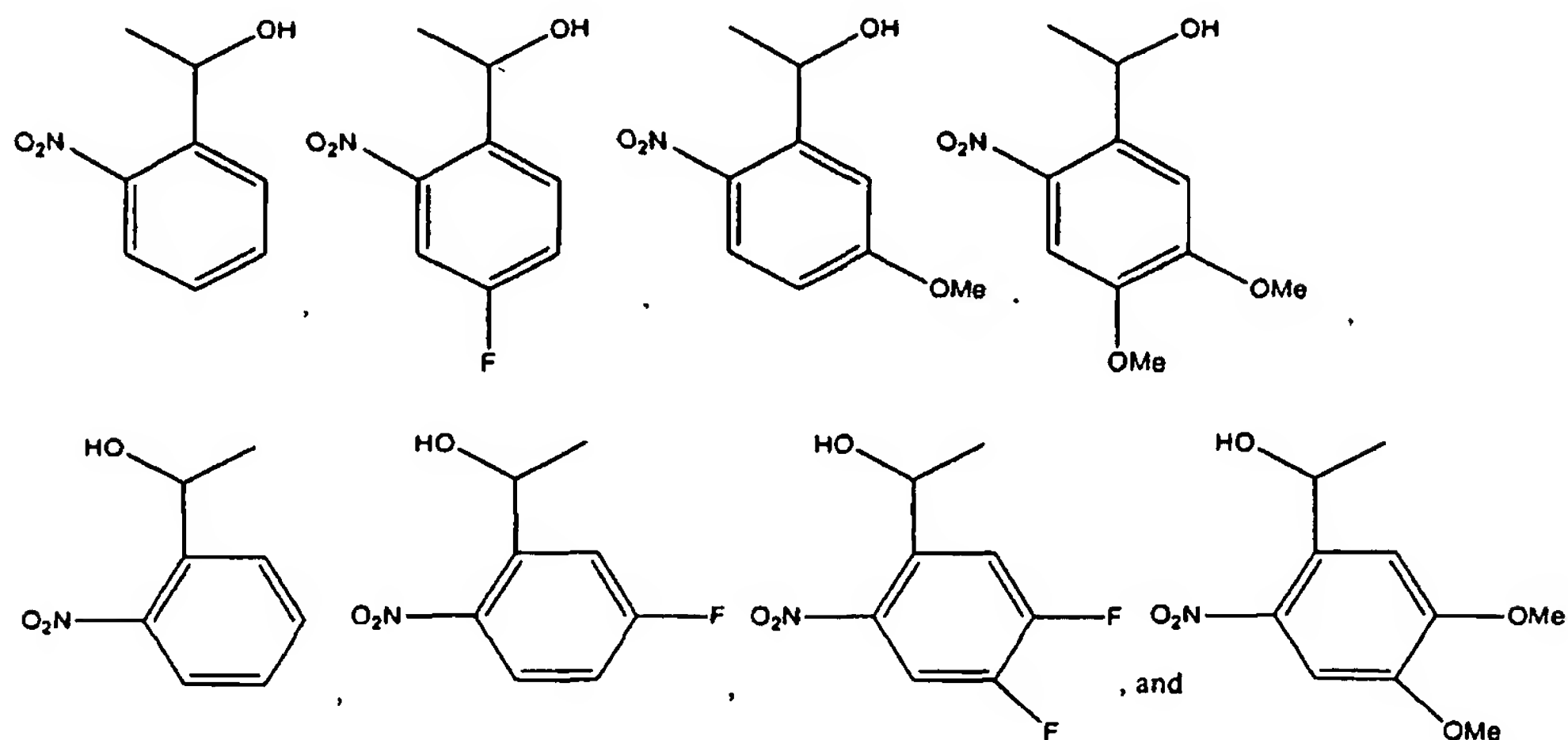
33. The method of claim 1, wherein the presence and size of any cleaved mass tag is determined by mass spectrometry.

34. The method of claim 33, wherein the mass spectrometry is selected from the group consisting of atmospheric pressure chemical ionization mass spectrometry, electrospray ionization mass

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spectrometry, and matrix assisted laser desorption ionization mass spectrometry.

35. The method of claim 1, wherein the target nucleic acid is a ribonucleic acid.

36. The method of claim 1, wherein the target nucleic acid is a deoxyribonucleic acid.

37. The method of claim 1, wherein the target nucleic acid is from a viral source.

38. A kit for simultaneously detecting in a sample the presence of one or more of a plurality of different target nucleic acids comprising a plurality of nucleic acid primers wherein (i) for each target nucleic acid at least one predetermined primer is used which is specific for that target nucleic acid, (ii) each primer has a mass tag of predetermined size bound thereto via a labile bond, and (iii) the mass tag bound to any primer specific for one target nucleic acid has a different mass than the mass tag bound to any primer specific for any other target nucleic acid.

39. A kit for simultaneously detecting in a sample the presence of one or more of a plurality of different target nucleic acids comprising (a) a plurality of nucleic acid primers wherein (i) for each target nucleic acid at least one predetermined primer is used which is specific for that target nucleic acid, (ii) each primer has a mass tag of predetermined size bound thereto via a labile bond, and (iii) the

mass tag bound to any primer specific for one target
nucleic acid has a different mass than the mass tag
bound to any primer specific for any other target
nucleic acid; and (b) a mass spectrometer.

40. A kit for simultaneously detecting in a sample the
presence of one or more of a plurality of different
target nucleic acids comprising (a) a plurality of
nucleic acid primers wherein (i) for each target
nucleic acid at least one predetermined primer is
used which is specific for that target nucleic acid,
(ii) each primer has a mass tag of predetermined
size bound thereto via a labile bond, and (iii) the
mass tag bound to any primer specific for one target
nucleic acid has a different mass than the mass tag
bound to any primer specific for any other target
nucleic acid, and (b) instructions for use.

41. A kit for simultaneously detecting in a sample the
presence of one or more of a plurality of different
target nucleic acids comprising (a) a plurality of
nucleic acid primers wherein (i) for each target
nucleic acid at least one predetermined primer is
used which is specific for that target nucleic acid,
(ii) each primer has a mass tag of predetermined
size bound thereto via a labile bond, and (iii) the
mass tag bound to any primer specific for one target
nucleic acid has a different mass than the mass tag
bound to any primer specific for any other target
nucleic acid; (b) a mass spectrometer; and (c)
instructions for simultaneously detecting in a
sample the presence of one or more of a plurality of

different target nucleic acids using the primers and
the mass spectrometer.

Mass Tag Precursor

Photoactive Mass Tag

1. HNO₃
2. NaBH₄

FIG. 1

FIG. 2

DNA Sequencing Using Solid Phase Capturable Terminator and MALDI-MS

FIG. 3

FIG. 4

FIG. 5

FIG. 6

ddCTP-11-Biotin

MW = 844

ddTTP-Linker I-11-Biotin

MW =977

ddATP-Linker II-11-Biotin

MW = 1,017

ddGTP-Linker III-11-Biotin

MW = 1,051

FIG. 7

FIG. 8

FIG. 9

$\lambda = 340\ nm$

(MW: 209.20)

(MW: 179.18)

(MW: 167.14)

(MW: 149.15)

FIG. 10

FIG 11.

FIG. 12

FIG. 13

FIG. 14

Fluorocarbon ions from ASGDI of perfluoro-dimethylcyclohexane on the Griffin MMS1

FIG. 15

FIG. 16

FIG. 17

FIG. 18

Specific Tag

FIG. 19

FIG. 20

FIG. 21

1. PCR amplification with Mass Tag primers   4. Automated sample injection, photocleavage

Cleavage of Mass Tags
from amplicons

Ultraviolet light

Purified samples

96-well thermocycler plate

2. Product purification on filter plate

5. Detection and pathogen identification

3. Elution into 96-well loading plate
for mass spectrometer analysis
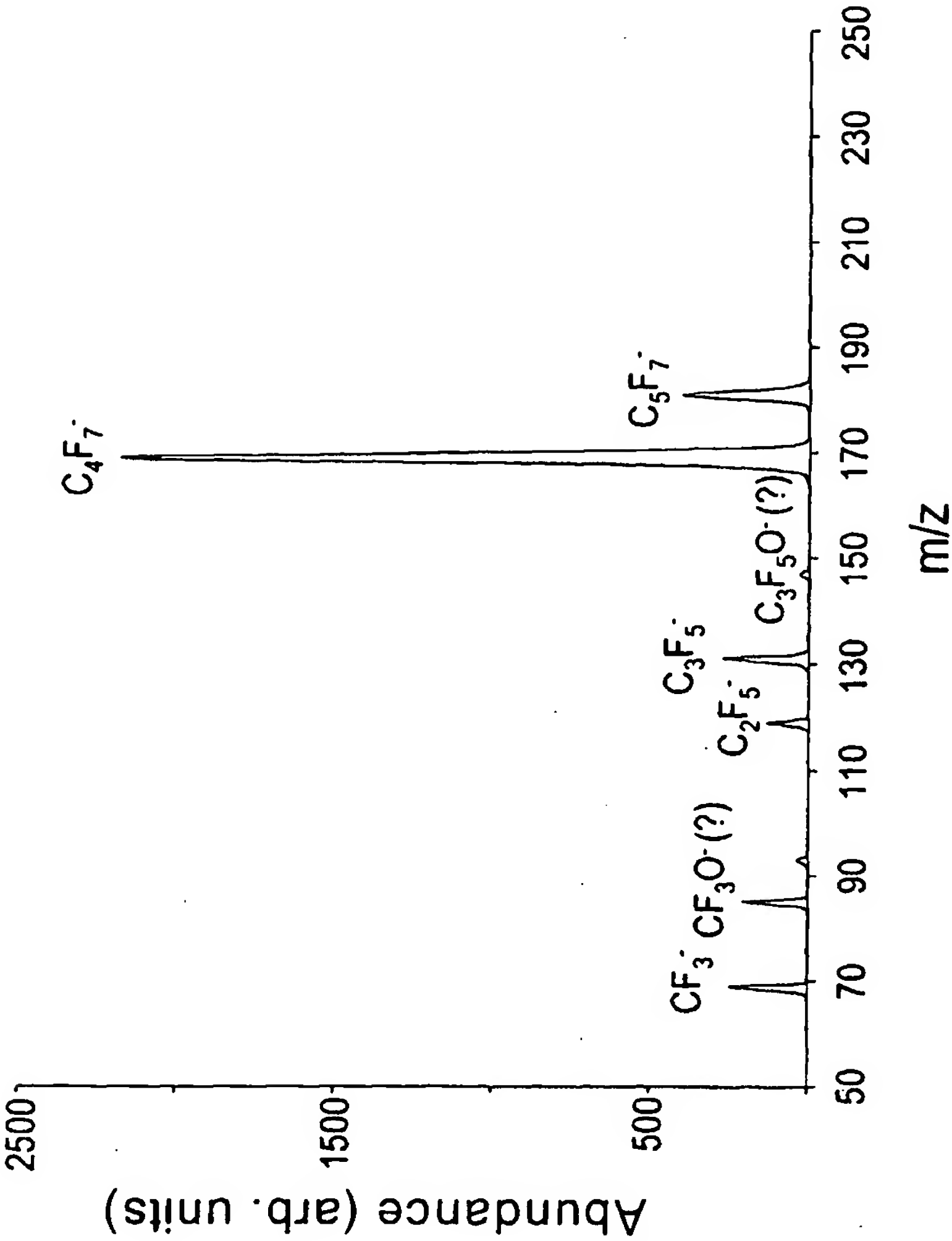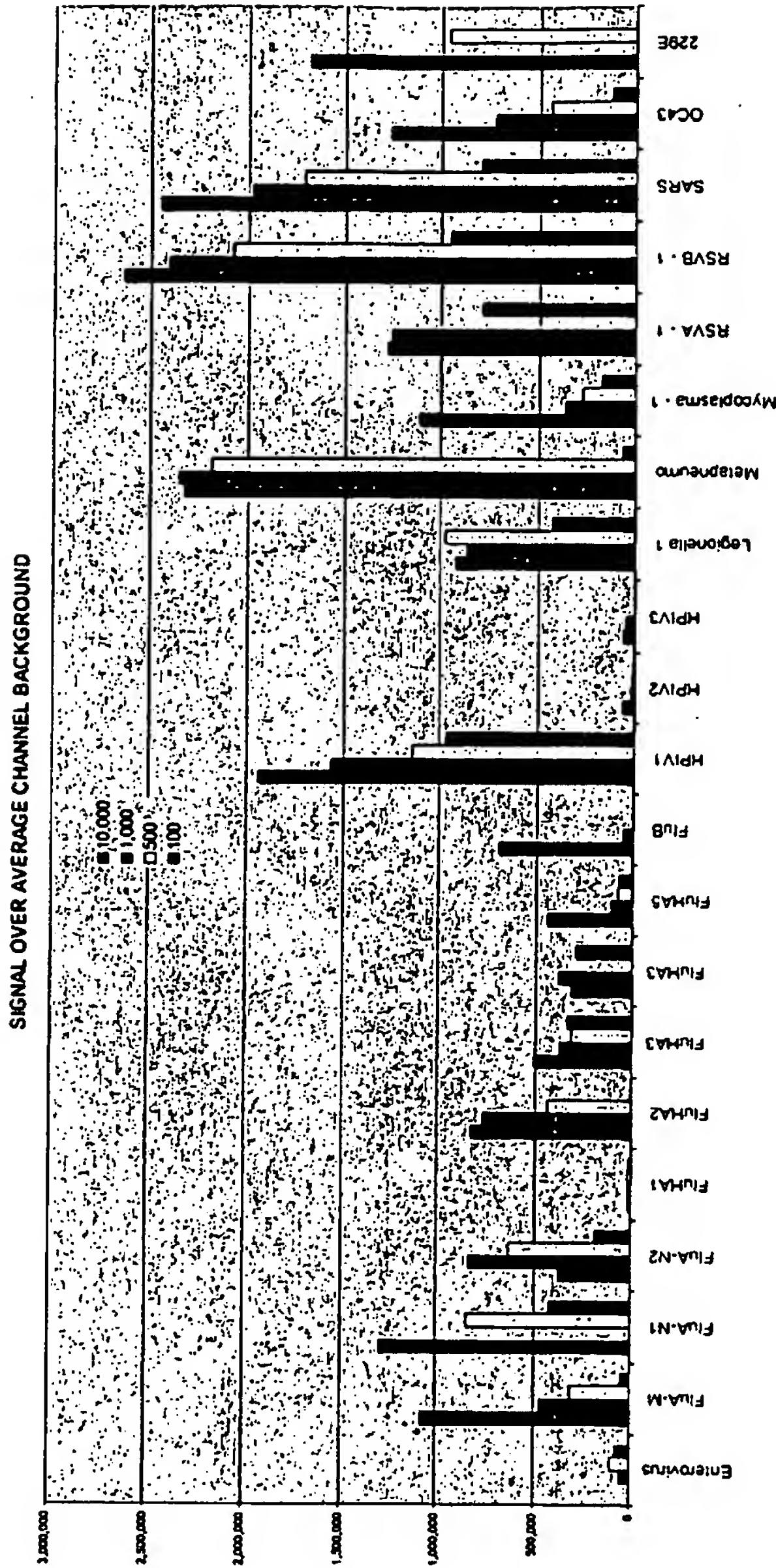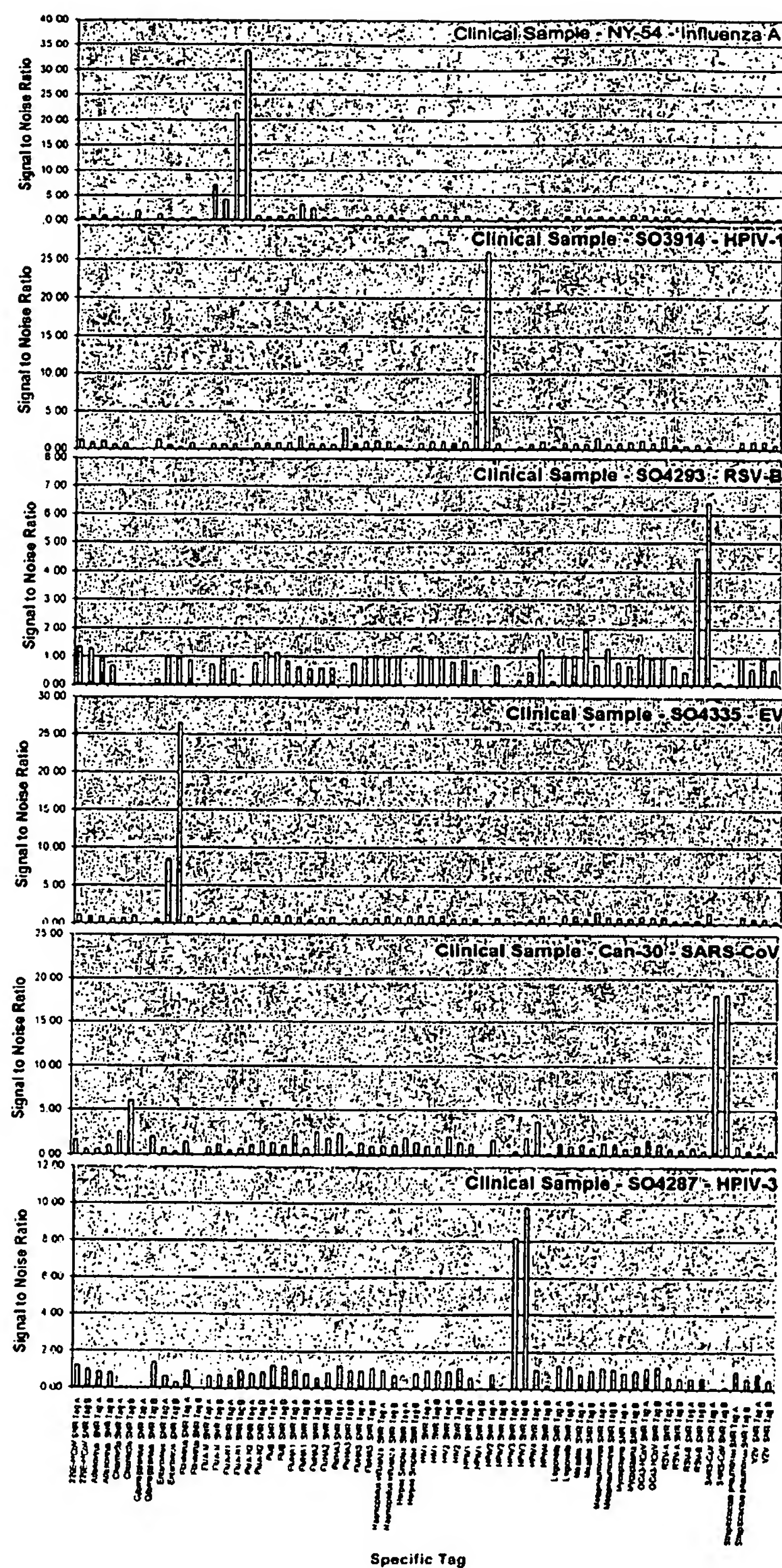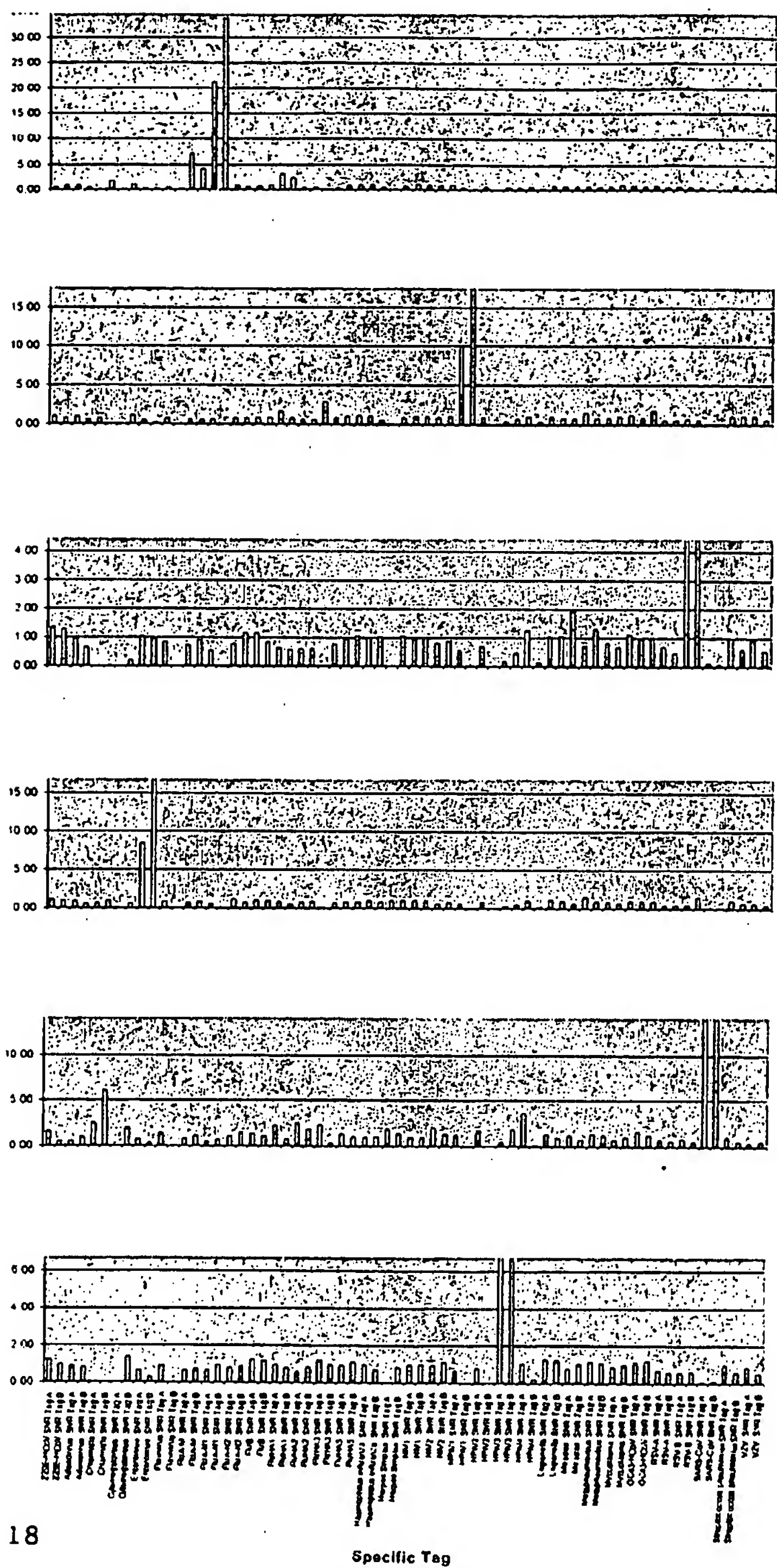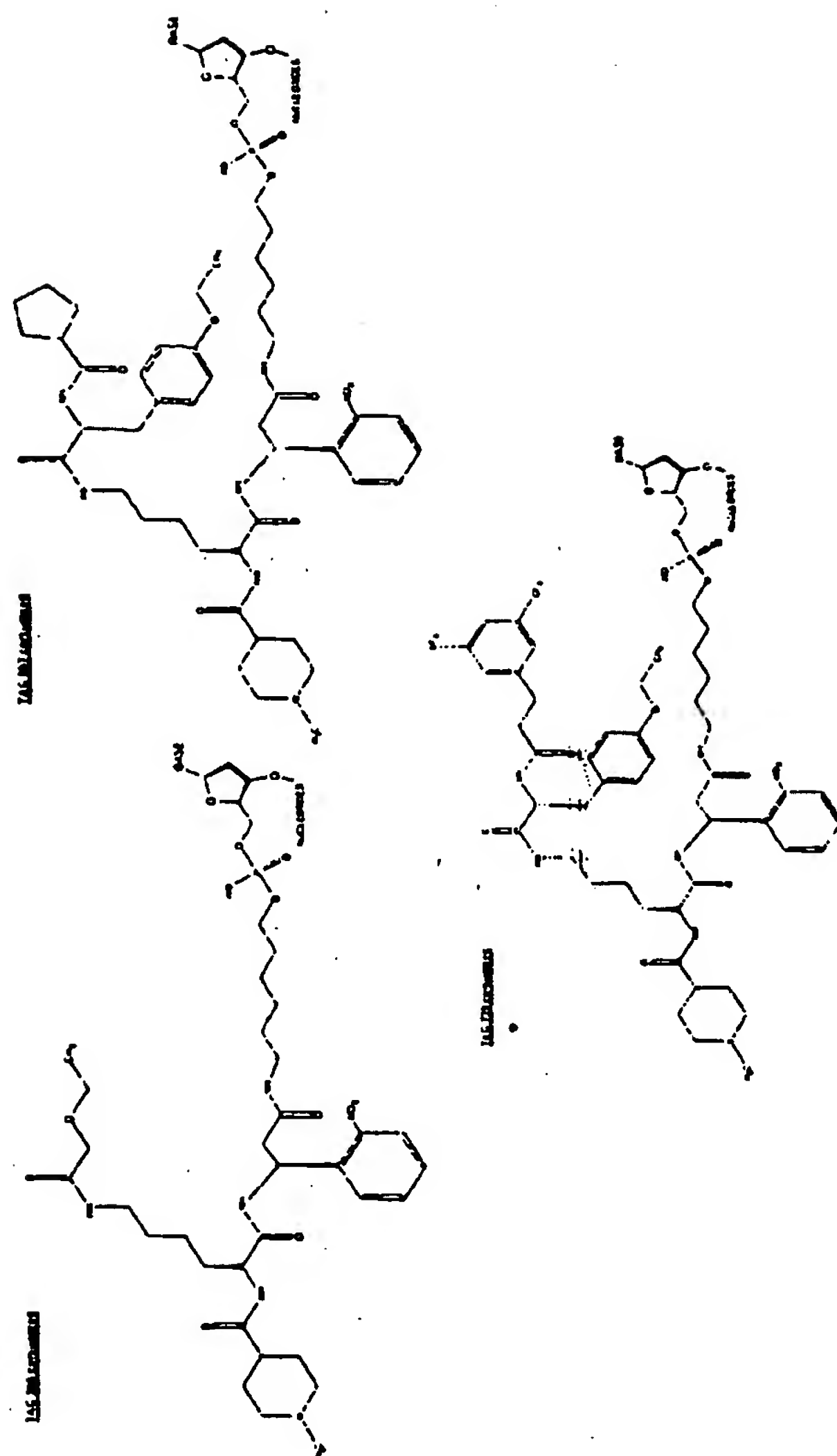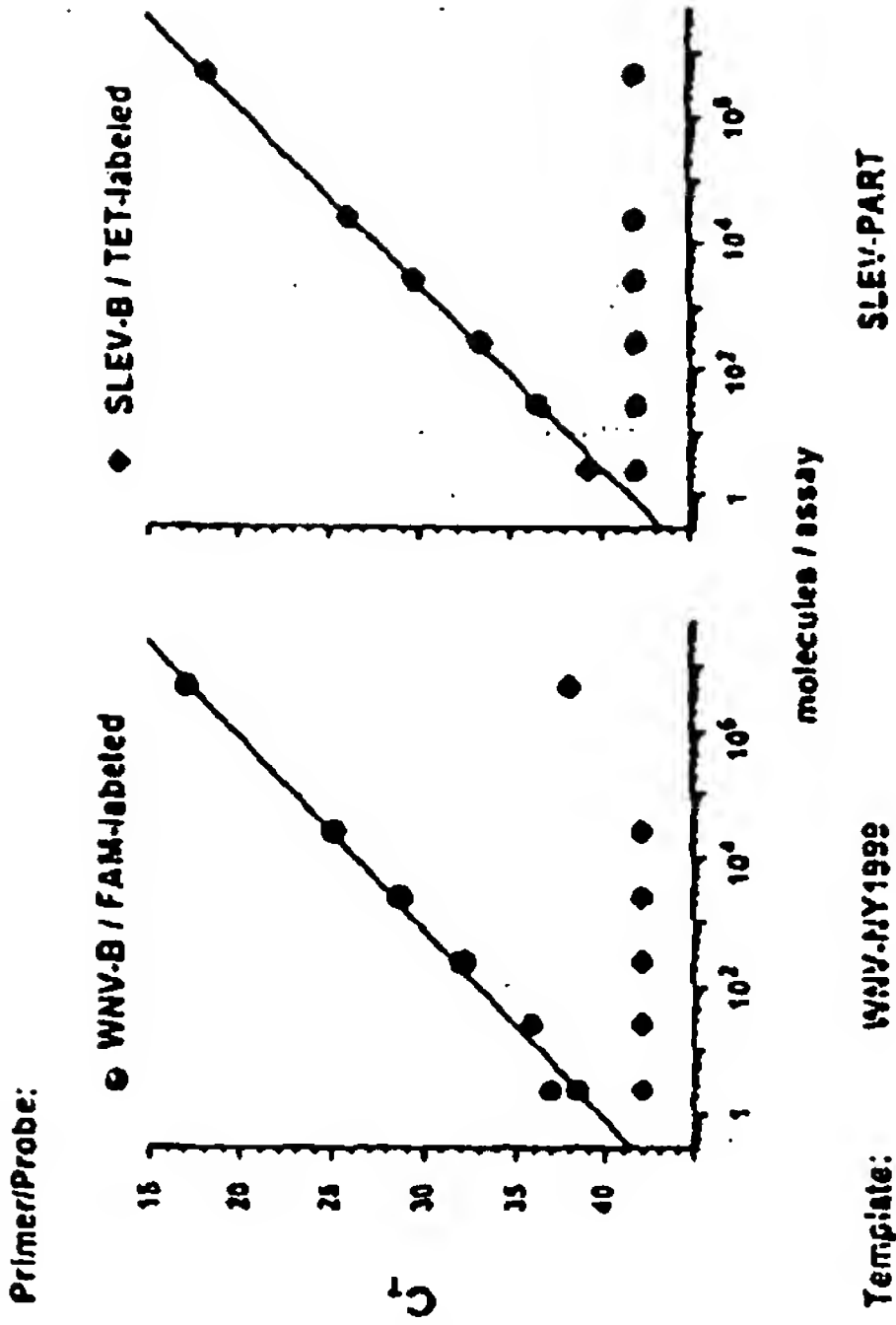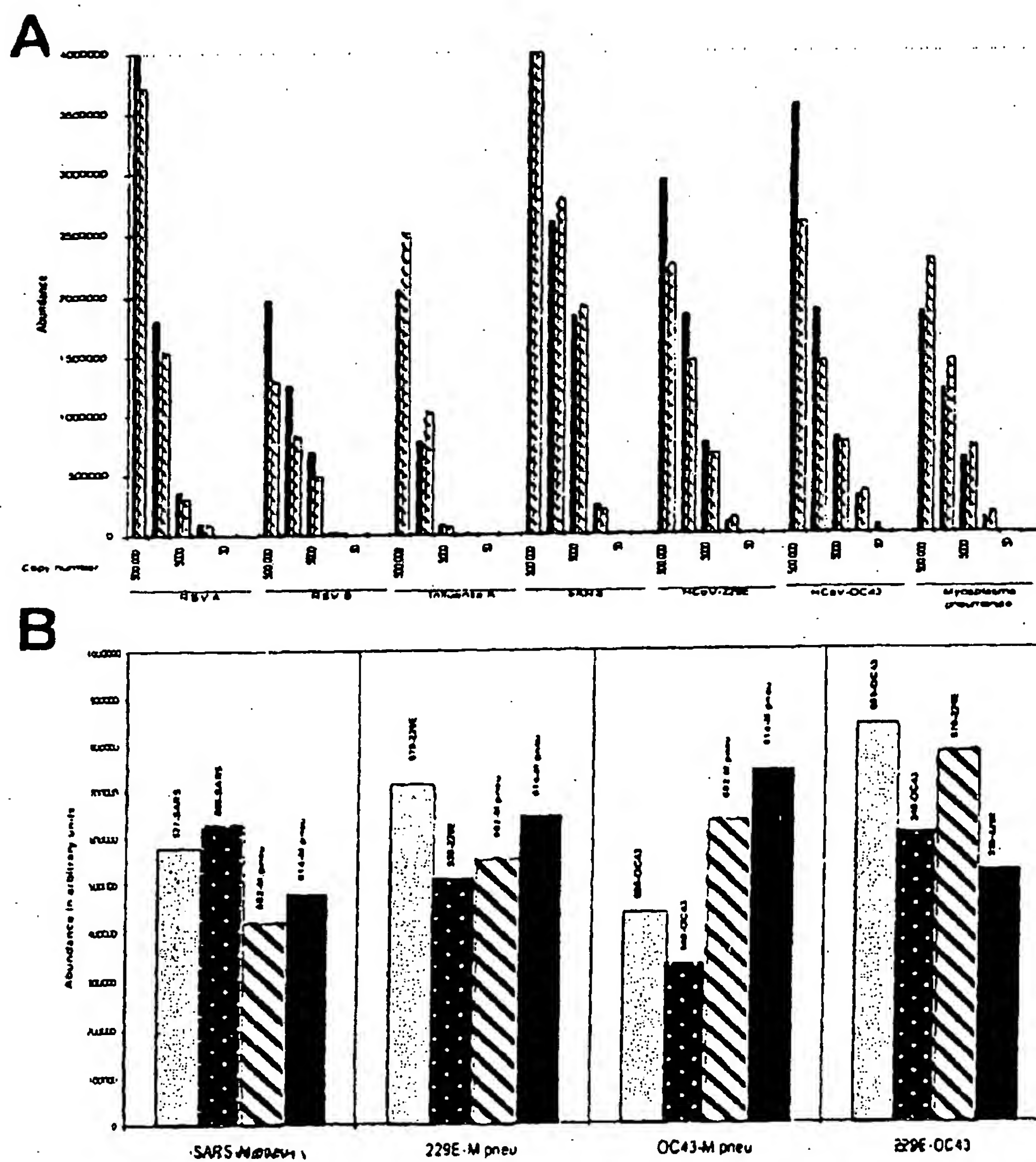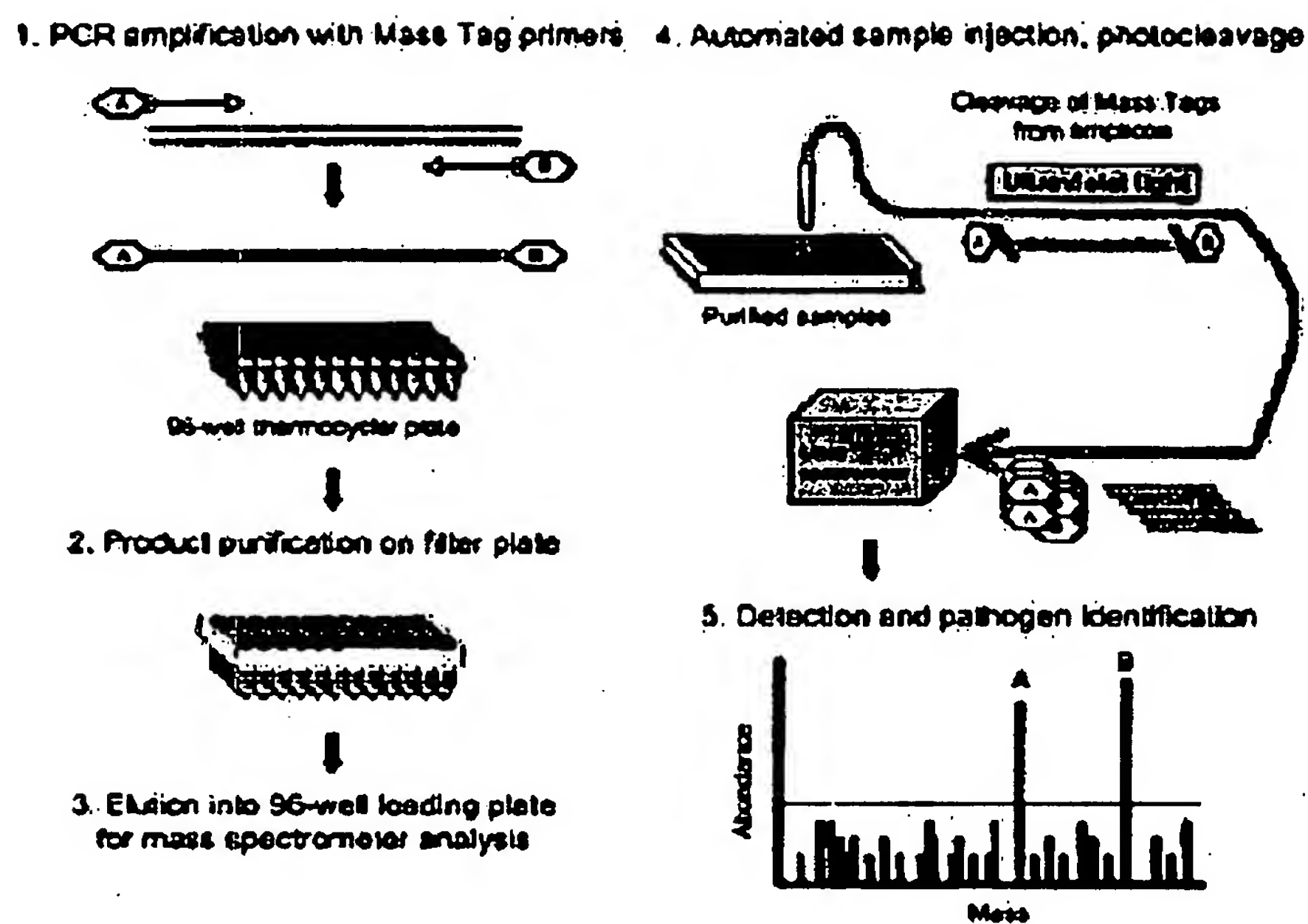
FIG. 22
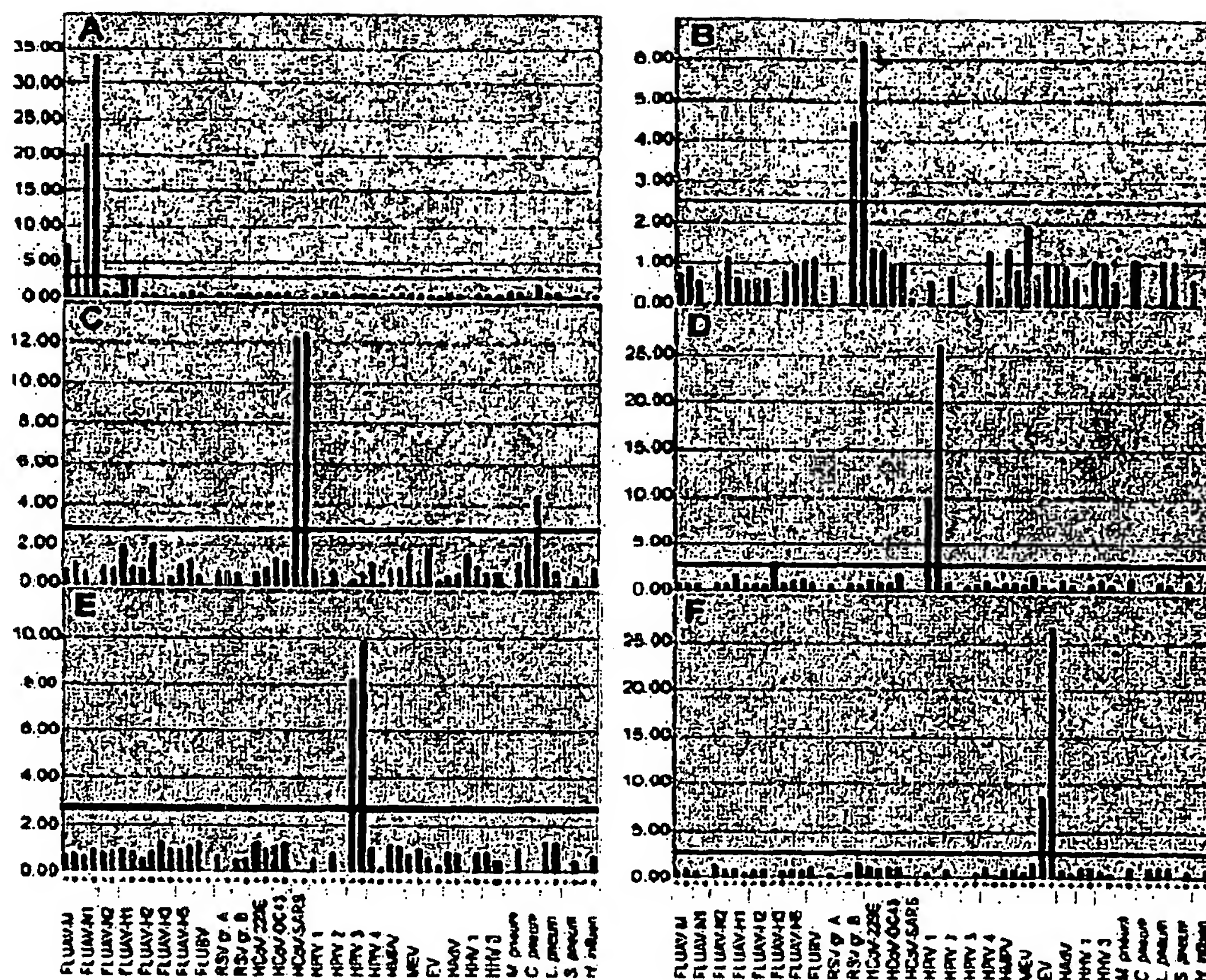
FIG. 23

SEQUENCE LISTING

<110> Lipkin, W. Ian

       Jingyue, Ju

       Thomas, Briese

<120> Mass Tag PCR For Multiplex Diagnostics

<130> 0575/71310-A

<160> 101

<170> PatentIn version 3.1

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Page 3

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<211> 26

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<211> 24

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<213> ARTIFICIAL SEQUENCE

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<211> 24

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<220>

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<210> 19

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<213>   ARTIFICIAL SEQUENCE


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<400>   19
agaccagagg gaaactatgc cc                                      22


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<213>   ARTIFICIAL SEQUENCE


<220>

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<210>   21

<211>   20

<212>   DNA

<213>   ARTIFICIAL SEQUENCE


<220>

<223>   FORWARD PRIMER FOR SARS CoV

<400>   21
aagcctcgcc aaaaacgtac                                         20


<210>   22

<211>   20

<212>   DNA

<213>   ARTIFICIAL SEQUENCE
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<223>   REVERSE PRIMER FOR SARS CoV

<400> 22
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<213>   ARTIFICIAL SEQUENCE

<220>

<223>   FORWARD PRIMER FOR 229E CoV

<400> 23
ggcgcaagaa ttcagaacca   20

<210>   24

<211>   19

<212>   DNA

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<223> REVERSE PRIMER TO 229E CoV

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taagagccgc agcaactgc   19

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<211>   21

<212>   DNA

<213>   ARTIFICIAL SEQUENCE

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<212>   DNA

<213>   ARTIFICIAL SEQUENCE

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<223>   REVERSE PRIMER FOR PARAINFLUENZA 1

<400>   32
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<210>   33

<211>   21

<212>   DNA

<213>   ARTIFICIAL SEQUENCE

<220>

<223>   FORWARD PRIMER FOR PARAINFLUENZA 2

<400>   33
ggacttggaa caagatggcc t   21

<210>   34

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<400>   34
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<210> 29

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<212> DNA

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<223> FORWARD PRIMER FOR METAPNEUMOVIRUS CANADIAN

<400> 29
aagtccaaag gcaggrctgt tatc   24

<210> 30

<211> 25

<212> DNA

<213> ARTIFICIAL SEQUENCE

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<223> REVERSE PRIMER FOR METAPNEUMOVIRUS CANADIAN

<400> 30
cctgaagcat trccaagaac aacac   25

<210> 31

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<212> DNA

<213> ARTIFICIAL SEQUENCE

<220>

<223> FORWARD PRIMER FOR PARAINFLUENZA 1

<400> 31
tacttttgac acatttagtt ccaggag   27

<210> 32

<211> 27

<223>   FORWARD PRIMER FOR OC43 CoV

<400>   25
tgtgcctatt gcaccaggag t   21

<210>   26

<211>   19

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<213>   ARTIFICIAL SEQUENCE

<220>

<223>   REVERSE PRIMER FOR OC43 CoV

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cccgatcgac aatgtcagc   19

<210>   27

<211>   24

<212>   DNA

<213>   ARTIFICIAL SEQUENCE

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<223>   FORWARD PRIMER FOR METAPNEUMOVIRUS EUROPEAN

<400>   27
aaccgtgtac taagtgatgc actc   24

<210>   28

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<212>   DNA

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<223>   REVERSE PRIMER FOR METAPNEUMOVIRUS EUROPEAN

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cattgtttga ccggccccat aa   22

<220>

<223> FORWARD PRIMER FOR PARAINFLUENZA 3

<400> 35
gctttcagac aagatggaac agtg   24

<210> 36

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<223> REVERSE PRIMER FOR PARAINFLUENZA 3

<400> 36
gcatkattga cccaatctga tcc   23

<210> 37

<211> 24

<212> DNA

<213> ARTIFICIAL SEQUENCE

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<223> FORWARD PRIMER FOR PARAINFLUENZA 4A

<400> 37
aacagaagga aatgatggtg gaac   24

<210> 38

<211> 20

<212> DNA

<213> ARTIFICIAL SEQUENCE

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<400> 38
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<210> 39

<211> 25

<212> DNA

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<223> FORWARD PRIMER FOR PARAINFLUENZA 4B

<400> 39
agaagaaaac aacgatgaga caagg   25

<210> 40

<211> 23

<212> DNA

<213> ARTIFICIAL SEQUENCE

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<400> 40
gtttccctgg ttcactctct tca   23

<210> 41

<211> 25

<212> DNA

<213> ARTIFICIAL SEQUENCE

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<223> FORWARD PRIMER FOR CYTOMEGALOVIRUS

<400> 41
tacagcacgc tcaacaccaa cgcct   25

<210> 42

<211> 25

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<223> REVERSE PRIMER FOR CYTOMEGALOVIRUS

<400> 42
cccggccttc accaccaacc gaaaa   25

<210> 43

<211> 25

<212> DNA

<213> ARTIFICIAL SEQUENCE

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<223> FORWARD PRIMER FOR MEASLES VIRUS

<400> 43
caagcatcat gatygccatt cctgg   25

<210> 44

<211> 26

<212> DNA

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<400> 44
cctgaatcyc tgcctatgat gggttt   26

<210> 45

<211> 18

<212> DNA

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<220>

<223>  FORWARD PRIMER FOR ADENOVIRUS

<400>  45
cccmttyaac caccaccg                                        18


<210>  46

<211>  20

<212>  DNA

<213>  ARTIFICIAL SEQUENCE


<220>

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<400>  46
acatccttbc kgaagttcca                                      20


<210>  47

<211>  28

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<213>  ARTIFICIAL SEQUENCE


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<223>  FORWARD PRIMER FOR ENTEROVIRUS

<400>  47
tcctccggcc cctgaatgcg gctaatcc                             28


<210>  48

<211>  25

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<213>  ARTIFICIAL SEQUENCE


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<400>  48
gaaacacggw cacccaaagt astcg                                    25


<210>  49

<211>  21

<212>  DNA

<213>  ARTIFICIAL SEQUENCE


<220>

<223>  FORWARD PRIMER FOR M. PNEUMONIAE

<400>  49
ccaaccaaac aacaacgttc a                                         21


<210>  50

<211>  20

<212>  DNA

<213>  ARTIFICIAL SEQUENCE


<220>

<223>  REVERSE PRIMER FOR M. PNEUMONIAE

<400>  50
accttgactg gaggccgtta                                          20


<210>  51

<211>  23

<212>  DNA

<213>  ARTIFICIAL SEQUENCE


<220>

<223>  FORWARD PRIMER FOR L. PNEUMOPHILAE

<400>  51
gcatwgatgt tartccggaa gca                                      23
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<210> 52

<211> 20

<212> DNA

<213> ARTIFICIAL SEQUENCE

<220>

<223> REVERSE PRIMER FOR L. PNEUMOPHILAE

<400> 52
cggttaaagc caattgagcg   20

<210> 53

<211> 20

<212> DNA

<213> ARTIFICIAL SEQUENCE

<220>

<223> FORWARD PRIMER FOR C. PNEUMONIAE

<400> 53
catggtgtca ttcgccaagt   20

<210> 54

<211> 20

<212> DNA

<213> ARTIFICIAL SEQUENCE

<220>

<223> REVERSE PRIMER FOR C. PNEUMONIAE

<400> 54
cgtgtcgtcc agccatttta   20

<210> 55

<211> 21

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<212>   DNA

<213>   ARTIFICIAL SEQUENCE


<220>

<223>   FORWARD PRIMER FOR RIFT VALLEY FEVER VIRUS

<400>   55
ggattgacct gtgcctgttg c                                              21


<210>   56

<211>   26

<212>   DNA

<213>   ARTIFICIAL SEQUENCE


<220>

<223>   REVERSE PRIMER FOR RIFT VALLEY FEVER VIRUS

<400>   56
gcattagaaa tgtcctcttt tgctgc                                         26


<210>   57

<211>   23

<212>   DNA

<213>   ARTIFICIAL SEQUENCE


<220>

<223>   FORWARD PRIMER FOR CCHF

<400>   57
agaacacgtg ccgcttacgc cca                                            23


<210>   58

<211>   27

<212>   DNA

<213>   ARTIFICIAL SEQUENCE
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<400> 58
ccattcytty ttraactcyt caaacca   27

<210> 59

<211> 27

<212> DNA

<213> ARTIFICIAL SEQUENCE

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<223> FORWARD PRIMER FOR EBOLA VIRUS

<400> 59
aacaccgggt cttaattctt atatcaa   27

<210> 60

<211> 28

<212> DNA

<213> ARTIFICIAL SEQUENCE

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<223> REVERSE PRIMER FOR EBOLA VIRUS

<400> 60
ggtggtaaaa ttcccatagt agttcttt   28

<210> 61

<211> 20

<212> DNA

<213> ARTIFICIAL SEQUENCE

<220>

<223> FORWARD PRIMER FOR MARBURG VIRUS

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<400>  61
ttccgtcaca agccgaaatt                                              20


<210>  62

<211>  29

<212>  DNA

<213>  ARTIFICIAL SEQUENCE


<220>

<223>  REVERSE PRIMER FOR MARBURG VIRUS

<400>  62
ttattttagt tgagaaaaga ggttcatgc                                   29


<210>  63

<211>  18

<212>  DNA

<213>  ARTIFICIAL SEQUENCE


<220>

<223>  FORWARD PRIMER FOR WEST NILE VIRUS

<400>  63
gctccgctgt ccctgtga                                               18


<210>  64

<211>  21

<212>  DNA

<213>  ARTIFICIAL SEQUENCE


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<223>  REVERSE PRIMER FOR WEST NILE VIRUS

<400>  64
cactctcctc ctgcatggat g                                           21
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<210> 65

<211> 22

<212> DNA

<213> ARTIFICIAL SEQUENCE

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<223> FORWARD PRIMER FOR ST. LOUIS ENCEPHALITIS VIRUS

<400> 65
catttgttca gctgtcccag tc   22

<210> 66

<211> 22

<212> DNA

<213> ARTIFICIAL SEQUENCE

<220>

<223> REVERSE PRIMER FOR ST. LOUIS ENCEPHALITIS VIRUS

<400> 66
ctcacccttc ccatgaattg ac   22

<210> 67

<211> 24

<212> DNA

<213> ARTIFICIAL SEQUENCE

<220>

<223> FORWARD PRIMER FOR HERPES SIMPLEX VIRUS

<400> 67
cccggatgcg gtccagacga ttat   24

<210> 68

<211> 24

<212> DNA

<213> ARTIFICIAL SEQUENCE

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<223> REVERSE PRIMER FOR HERPES SIMPLEX VIRUS

<400> 68
cccgcggagg ttgtacaaaa agct   24

<210> 69

<211> 25

<212> DNA

<213> ARTIFICIAL SEQUENCE

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<223> FORWARD PRIMER FOR HIV-1

<400> 69
ttcttggagc agcggaagca catgg   25

<210> 70

<211> 25

<212> DNA

<213> ARTIFICIAL SEQUENCE

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<223> REVERSE PRIMER FOR HIV-1

<400> 70
ttmatgcccc agacgtagtt caaca   25

<210> 71

<211> 19

<212> DNA

<213> ARTIFICIAL SEQUENCE

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<220>

<223>   FORWARD PRIMER FOR HIV-2

<400>   71
ggctgcacgc cctatgata                                                    19


<210>   72

<211>   19

<212>   DNA

<213>   ARTIFICIAL SEQUENCE


<220>

<223>   REVERSE PRIMER FOR HIV-2

<400>   72
tctgcatggc tgcttgatg                                                    19


<210>   73

<211>   18

<212>   DNA

<213>   ARTIFICIAL SEQUENCE


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<223>   FORWARD PRIMER FOR N. MENIGITIDIS

<400>   73
tctgaagcca ttggccgt                                                     18


<210>   74

<211>   18

<212>   DNA

<213>   ARTIFICIAL SEQUENCE


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<223>   REVERSE PRIMER FOR N. MENIGITIDIS

<400>   74
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caaacacacc acgcgcat   18

<210> 75

<211> 22

<212> DNA

<213> ARTIFICIAL SEQUENCE

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<223> FORWARD PRIMER FOR S. PNEUMONIAE

<400> 75
agcgatagct ttctccaagt gg   22

<210> 76

<211> 23

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<223> REVERSE SEQUENCE FOR S. PNEUMONIAE

<400> 76
cttagccaac aaatcgttta ccg   23

<210> 77

<211> 27

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<223> FORWARD PRIMER FOR H. INFLUENZAE

<400> 77
aagctccttg mattttttgt attagaa   27

<210> 78

<211>   23

<212>   DNA

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<223>   REVERSE PRIMER FOR H. INFLUENZAE

<400>   78
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<210>   79

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<223>   FORWARD PRIMER FOR INFLUENZA B

<400>   79
agaccagagg gaaactatgc cc   22

<210>   80

<211>   24

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<223>   REVERSE PRIMER FOR INFLUENZA B

<400>   80
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<210>   81

<211>   20

<212>   DNA

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<223>   FORWARD PRIMER DIRECTED TO SARS CoV

<400>   81
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<210>   82

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<223>, REVERSE PRIMER DIRECTED TO SARS CoV

<400>   82
aagtcagcca tgttcccgaa   20

<210>   83

<211>   20

<212>   DNA

<213>   ARTIFICIAL SEQUENCE

<220>

<223>   FORWARD PRIMER DIRECTED TO 229E-CoV

<400>   83
ggcgcaagaa ttcagaacca   20

<210>   84

<211>   19

<212>   DNA

<213>   ARTIFICIAL SEQUENCE

<220>

<223>   REVERSE PRIMER DIRECTED TO 229E-CoV

<400>   84
taagagccgc agcaactgc   19

<210>   85

<211>   21

<212>   DNA

<213>   ARTIFICIAL SEQUENCE

<220>

<223>   FORWARD PRIMER FOR OC43 CoV

<400>   85
tgtgcctatt gcaccaggag t   21

<210>   86

<211>   19

<212>   DNA

<213>   ARTIFICIAL SEQUENCE

<220>

<223>   REVERSE PRIMER FOR OC43 CoV

<400>   86
cccgatcgac aatgtcagc   19

<210>   87

<211>   25

<212>   DNA

<213>   ARTIFICIAL SEQUENCE

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<223>   FORWARD PRIMER FOR CYTOMEGALOVIRUS

<400>   87
tacagcacgc tcaacaccaa cgcct   25

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<210>  88

<211>  25

<212>  DNA

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<400>  88
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<210>  89

<211>  23

<212>  DNA

<213>  ARTIFICIAL SEQUENCE


<220>

<223>  FORWARD PRIMER FOR VARICELLA ZOSTER VIRUS

<400>  89
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<210>  90

<211>  23

<212>  DNA

<213>  ARTIFICIAL SEQUENCE


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<223>  REVERSE PRIMER FOR VARICELLA ZOSTER VIRUS

<400>  90
tcgctatgtg ctaaaacacg cgg                                          23


<210>  91

<211>  25
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<212>  DNA

<213>  ARTIFICIAL SEQUENCE


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<223>  FORWARD PRIMER FOR MEASLES VIRUS

<400>  91
caagcatcat gatygccatt cctgg                                    25


<210>  92

<211>  26

<212>  DNA

<213>  ARTIFICIAL SEQUENCE


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<223>  REVERSE PRIMER FOR MEASLES VIRUS

<400>  92
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<210>  93

<211>  18

<212>  DNA

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<220>

<223>  REVERSE PRIMER FOR ADENOVIRUS

<400>  93
cccmttyaac caccaccg                                            18


<210>  94

<211>  20

<212>  DNA

<213>  ARTIFICIAL SEQUENCE
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<220>

<223> REVERSE PRIMER FOR ADNEOVIRUS

<400> 94
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<210> 95

<211> 28

<212> DNA

<213> ARTIFICIAL SEQUENCE

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<223> FORWARD PRIMER FOR ENTEROVIRUS

<400> 95
tcctccggcc cctgaatgcg gctaatcc   28

<210> 96

<211> 25

<212> DNA

<213> ARTIFICIAL SEQUENCE

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<223> REVERSE PRIMER FOR ENTEROVIRUS´

<400> 96
gaaacacggw cacccaaagt astcg   25

<210> 97

<211> 19

<212> DNA

<213> Artificial Sequence

<220>

<223> Primer directed to SARS virus

<400> 97
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<210> 98

<211> 25

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<223> Forward Primer for Enterovirus A/B 702/495

<400> 98
tccggcccct gaatgcggct aatcc   25

<210> 99

<211> 20

<212> DNA

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<220>

<223> Forward Primer for Enterovirus A/B 702/495

<400> 99
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<210> 100

<211> 22

<212> DNA

<213> Artificial Sequence

<220>

<223> Foward primer for S. Pneumoniae

<400> 100
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<210> 101

<211> 23

<212> DNA

<213> Artificial Sequence

<220>

<223> Reverse primer for S. Pneumoniae

<400> 101
cttagccaac aaatcgttta ccg   23

Attachment A

1) Page 1; lines 5-7
2) Page 12; lines 9-10
3) Page 13; line 4
4) Page 17; line 3 to Page 22; line 4
5) Page 83; lines 7-8
6) Pages 96-112
7) Page 119; claim 38 to Page 121; claim 41
8) Page 122
9) Figure 23

IDS REFERENCES

☐ FOR